Testing two ageing theories in *Caenorhabditis elegans*

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PhD thesis

Declaration

Hereby I confirm that the work presented in this thesis was conducted by myself if not stated otherwise. Any information derived from other sources has been referenced accordingly.

…….
Sara Valentini

Abstract

In my thesis I was testing two established ageing theories in *C. elegans*. One was about the role of oxidative damage, induced via the Fenton reaction, in *C. elegans* ageing. In my other project I was investigating the role of sirtuins, NAD^+- dependent histone deacetylases, in ageing.

The oxidative damage theory predicts that reactive oxygen species (ROS) is a main cause of ageing. Iron can generate ROS via the Fenton reaction, indicating that iron homeostasis might protect against aging. Ferritins, iron storage proteins, regulate the iron concentration by storing excess iron. *C. elegans* has two ferritin genes, *ftn-1* and *ftn-2*. Long-lived *daf-2* mutants show an increase in *ftn-1* mRNA levels, indicating that *ftn-1* might contribute to longevity assurance. I tested the role of *ftn-1* in longevity assurance and found that reduced *ftn-1* levels did not affect *daf-2* mutant longevity or wildtype life span, nor did over-expression of *ftn-1* increase life span. Changing iron levels via *ftn-1* over-expression or iron chelator treatment led to resistance to oxidative stress, but had no effect on ageing. Overall, our results show that ferritin does not contribute to longevity assurance, and imply that oxidative damage, induced via the Fenton reaction is not a determinant of aging in *C. elegans*.

Over-expression of sirtuins has been reported to increase life span in yeast, *C. elegans* and *Drosophila*. Rumours and contradictory findings caused us to re-test the effects of sirtuin over-expression on ageing. We found that backcrossing the two mainly used *sir-2.1* over-expressing strains LG100 and NL3909, to wildtype background abolished the increase in life span, without changing the over-expression of *sir-2.1.* Reducing *sir-2.1* levels had no effect on LG100. Instead, longevity co-segregated with a second-site mutation affecting sensory neurons in LG100. These findings question the role of sirtuins in ageing.

Dedicated to
Mom, Dad and Manu
Without your help, support, patience and love
I would have never made it.

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List of Figures and Tables 10

Chapter 1: Introduction 16

1.1. Ageing 16

1.2. Possible explanations for ageing 16
1.2.1. Energy metabolism and ageing 17
1.2.2. Damage theory of ageing 18
1.2.2.1. The oxidative damage theory of ageing 19
1.2.2.2. Oxidative damage in biological systems 20
1.2.2.3. Heavy metals causing oxidative damage 22
1.2.2.4. The role of iron in oxidative damage 24
1.2.2.5. Defense mechanisms to oxidative damage in biological systems 24
1.2.2.5.1. Enzymatic antioxidants 25
1.2.2.5.2. Ferritins in vertebrates 26
1.2.2.6. Iron chelators 31

1.3. Mechanisms of ageing 31
1.3.1. Dietary Restriction (DR) 31
1.3.2. Single-gene effect on life span 33
1.3.3. The insulin IGF-1 signaling (IIS) pathway and ageing 34

1.4. Model organisms in ageing research 35
1.4.1. *C. elegans* as a model organism for studying ageing 36
1.4.2. *C. elegans* genetic terminology 37
1.4.3. Common *C. elegans* mutant classes 38
1.4.4. *C. elegans* anatomy 39
1.4.4.1. *C. elegans* hermaphrodites 40
1.4.4.2. *C. elegans* males 41
1.4.4.3. *C. elegans* nervous system 42
1.4.4.3.1. Sensory neurons 43
1.4.4.3.1.1. Amphid and phasmid neurons 44
1.4.4.4. Dye-filling defective (Dyf) phenotype 46
1.4.5. *C. elegans* life cycle 47
1.4.6. Dauer larvae 48
1.4.6.1. Mutations in abnormal dauer formation (*daf*) genes 50
1.4.7. The insulin IGF-1 signaling (IIS) pathway in *C. elegans* 50
1.4.8. Effect of dye-filling (dyf) defective mutations on *C. elegans* life span 53
1.4.8.1. The Dyf phenotype is not caused by DR 54
1.4.9. Antioxidants in *C. elegans* 54
1.4.9.1. Ferritins in *C. elegans* 55
1.4.9.2. Ferritin regulation in *C. elegans* 56
1.4.9.3. The role of ferritins in oxidative damage in *C. elegans* 58
1.4.10. Agents used to induce oxidative stress in *C. elegans* 59
1.4.10.1. Hyperoxia 59
1.4.10.2. Redox cycling agents 59
1.4.10.3. Peroxides 60

1.5. Arguments against the oxidative damage theory 60
1.5.1. Antioxidant defense and ageing in *C. elegans* 62
1.5.2. Antioxidants and ageing in long-lived IIS mutants 63

1.6. Sirtuins and their role in ageing 65
1.6.1. Histone deacetylases (HDAC) 66
1.6.1.1. Classes of histone deacetylases (HDAC) 67
1.6.1.2. Chromatin silencing mechanism 68
1.6.1.3. Catalytic mechanism of sirtuins 69
1.6.2. The role of sirtuins in ageing in different model organisms 71
1.6.2.1. How sirtuins became a major topic in biogerontology 71
1.6.2.2. Effects of sirtuins on yeast ageing are context specific 72
1.6.2.3. The role of sirtuins in *Drosophila* life span 73

1.6.2.4. The role of sirtuins in *C. elegans* ageing 73
1.6.2.4.1. *sir-2.1* acts downstream of *daf-16* in longevity assurance 74
1.6.3. Do sirtuins mediate the effects of dietary restriction (DR) on life span? 75
1.6.3.1. Sirtuins and their involvement in DR effects in yeast 75
1.6.3.2. Findings questioning the role of sirtuins in DR effects in yeast 76
1.6.3.3. The role of sirtuins in the DR response in *Drosophila* 77
1.6.3.4. The role of sirtuins in the DR response in *C. elegans* 78
1.6.3.5. The role of sirtuins in mammalian DR responses 78
1.6.4. The resveratrol story: sirtuin activator or not? 79
1.6.4.1. Findings arguing against resveratrol as a sirtuin activator 81
1.6.4.2. Resveratrol does not increase life span in a sirtuin dependent manner 82
1.6.5. The hyping of sirtuins to the public 83

Chapter 2: Material & Methods 86

2.1. Materials 86
2.1.1. Reagents 86
2.1.2. *C. elegans* strains used 88

2.2. Methods 89
2.2.1. Microscopy 89
2.2.2. Maintenance of stocks 89
2.2.2.1. Maintenance of extrachromosomal transgenic lines 90
2.2.3. Decontamination and synchronization of nematode stocks 90
2.2.3.1. Alkaline hypochlorite treatment (egg prep) 91
2.2.3.2. Egg lay 91
2.2.4. Life span measurements 92
2.2.5. GFP measurement 92
2.2.6. Stress assays 93
2.2.6.1. Iron treatments 93
2.2.6.2. Oxidative stress assays 93
2.2.6.3. High temperature induction of constitutive dauer formation (Hid) assay 94
2.2.7. Statistics used 94
2.2.8. Dye-filling defect (Dyf) test 95
2.2.9. RNAi by ingestion of dsRNA 95
2.2.9.1. Mechanism 95
2.2.9.2. RNAi feeding protocol 96
2.2.10. Generation of transgenic worms via microinjection 97
2.2.10.1. Amplification of the *ftn-1* fragment 97
2.2.10.2. Preparation of the Marker 98
2.2.10.3. Further preparations for injection 99
2.2.10.4. The injection process 99
2.2.11. Classical genetic methods 101
2.2.11.1. *C. elegans* conventions 101
2.2.11.2. Generation of males 102
2.2.11.3. Crosses 103
2.2.11.4. Mapping 104
2.2.11.4.1. Two-point mapping 104
2.2.11.4.2. Three-point mapping 105
2.2.12. Molecular biology 110
2.2.12.1. RNA extraction 110
2.2.12.2. First strand cDNA synthesis 111
2.2.12.3. Real-Time (RT) polymerase chain reaction (PCR) 112
2.2.13. Biochemistry 113
2.2.13.1. Protein gels 113
2.2.13.2. Protein sample collection 114
2.2.13.3. Protein extraction 114
2.2.13.4. Preparation of oxyblot samples 115
2.2.13.5. Western blotting 115
2.2.14. Measuring free iron levels using electron paramagnetic resonance (EPR) spectroscopy 116
2.2.14.1. The theory behind the method 116

2.2.14.2. Sample preparation 117
2.2.14.3. EPR spectroscopy 118

Chapter 3: Investigating the role of oxidative damage in C. elegans ageing 120

3.1. Investigating the role of ferritin in *daf-2* longevity 120
3.1.1. Why are we interested in *daf-2* mutants? 120
3.1.2. The discovery of elevated *ftn-1* levels in *daf-2* mutants 121
3.1.3. Aims 122

3.2. Results 123
3.2.1. *ftn-1* does not contribute to *daf-2* mutant longevity 123
3.2.2. *ftn-1* protects against oxidative stress 126
3.2.3. Effect of increasing iron levels 130
3.2.3.1. Effect of iron supplementation on free iron levels *in vivo* 131
3.2.3.2. Effect of increased external iron levels on *ftn-1* expression 135
3.2.3.3. Iron supplementation increases oxidative damage 137
3.2.4. Iron supplementation causes hypersensitivity to peroxide toxicity in *C. elegans* 140
3.2.5. Iron supplementation reduces *C. elegans* life span 143
3.2.6. Effect of iron supplementation on ageing in *C. elegans* 148
3.2.7. Effect of an iron chelator on free iron levels in *C. elegans* 151
3.2.7.1. Effect of deferoxamine on free iron levels in *C. elegans* 152
3.2.7.2. Effect of deferoxamine on *Pftn-1::GFP* expression 154
3.2.8. Effect of deferoxamine on peroxide resistance 156
3.2.8.1. Effect of deferoxamine on oxidative damage in *C. elegans* 159
3.2.9. Effect of deferoxamine on *C. elegans* ageing 161

3.3. Discussion 165
3.3.1. *ftn-1* is not important in *daf-2* mutant longevity 165
3.3.2. *ftn-1* contributes weakly to resistance to peroxide stress 166
3.3.3. Effects of iron supplementation on *C. elegans* 167
3.3.4. Effect of reduced iron levels in *C. elegans* 168

Chapter 4: Effect of over-expressing ftn-1 on oxidative damage and ageing in C. elegans 170

4.1. Introduction 170
4.1.1. Generation of *ftn-1* over-expressing lines 170
4.1.2. The *coel::GFP* marker 171

4.2. Results 173
4.2.1. Selection of *ftn-1* over-expressing (OE) lines 173
4.2.2. Phenotypic effects of *ftn-1* over-expression 177
4.2.2.1. *ftn-1* OE does not reduce brood size 178
4.2.2.2. *ftn-1* OE appears to reduce free iron levels 179
4.2.2.3. *ftn-1* OE does not reduce oxidative damage levels 182
4.2.2.4. Effect of *ftn-1* OE on oxidative damage levels during iron stress 185
4.2.2.5. *ftn-1* OE does not protect against iron supplement toxicity 187
4.2.2.6. *ftn-1* OE increases resistance to *tert*-butyl hydroperoxide (TBH) 190
4.2.2.7. *ftn-1* OE has no effect on ageing 194

4.3. Discussion 198
4.3.1. The effect of *wuEx187* on free iron levels 198
4.3.2. The effect of *wuEx187* on oxidative damage levels 199
4.3.3. The effect of *wuEx187* on resistance to oxidative stress 201
4.3.4. The effect of *wuEx187* on organismal ageing 201
4.3.5. Conclusion 202

Chapter 5: The effect of sir-2.1 over-expression on 204

C. elegans life span in the transgenic line LG100 204

5.1. Introduction 204
5.1.1. A chromosomal duplication including *sir-2.1* increases life span 204

5.1.2. Creation of LG100 205

5.2. Results 207
5.2.1. The *sir-2.1* over-expressing line LG100 is long-lived 207
208
5.2.2. Effect of backcrossing in the *sir-2.1* over-expresser strain LG100 210
5.2.2.1. Out-crossing of LG100 does not affect *sir-2.1* over-expression 215
5.2.3. Does a deleterious second site mutation suppress the effects of *sir-2.1* on life span in GA468 217
5.2.4. Effect of reduced *sir-2.1* expression levels in LG100 219
5.2.5. A dye-filling defect (Dyf) mutation causes the life span extension in LG100 223
5.2.6. Mapping the *dyf-?* locus 232
5.2.6.1. Two point mapping 232
5.2.6.2. Three-point mapping 234
5.2.6.3. Testing *dyf-1(nm335)* for *dyf-?(wu250)* identity 240
5.2.7. Verifying the effect on life span of the duplication *mDp4* 241
5.2.7.1. Life span of *mDp4* duplication strain is increased upon reduced *sir-2.1* expression 247

5.3. Discussion 251
5.3.1. LG100 longevity is attributable to *dyf-?(wu250) I* 251
5.3.2. *E. coli* food source influences effect of *dyf-?(wu250)* on life span 252
5.3.3. Variability among *rol-6* control lines in life span 253
5.3.4. Conclusions 254

Chapter 6: The effect of sir-2.1 over-expression on 255

C. elegans life span in the transgenic line NL3909 255

6.1. Introduction 255
6.1.1. Generation of a new *sir-2.1* over-expressing line 255
6.1.2. Effect of *sir-2.1* over-expression in *C. elegans* 256

6.2. Results 259
6.2.1. The *sir-2.1* over-expressing line NL3909 is long-lived 259
6.2.2. Effect of backcrossing on the *sir-2.1* over-expresser strain NL3909 262
6.2.3. GA905 is competent for life extension 268
6.2.4. Effect of reduced *sir-2.1* expression levels in NL3909 269
6.2.5. Testing NL3909 for a second site *daf-c* mutation 274
6.2.6. Is *daf-?* mutation in DP38 sufficient to increase life span in NL3909? 277

6.3. Discussion 281
6.3.1. The sirtuin controversy 281
6.3.2. Over-expression of *sir-2.1* does not increase *C. elegans* life span 282
6.3.3. Perspectives of the sirtuin affair 286

Conclusion 288

Bibliography 290

Appendix: Burnett et al., 2011 316

List of Figures and Tables

Chapter 1

Page

Figures

Figure 1.1: List of redox reactions showing the.. 20
Figure 1.2: Image of ferritin protein with its different subunits 27
Figure 1.3: Ferroxidase reaction of ferritin 28
Figure 1.4: Ferritin regulation in vertebrates 30
Figure 1.5: Naming convention for the transgenic.. 38
Figure 1.6: *C. elegans* hermaphrodite anatomy 40
Figure 1.7: *C. elegans* male anatomy 41
Figure 1.8: Nervous system of *C. elegans* 42
Figure 1.9: Structure of amphids 45
Figure 1.10: Life cycle of *C. elegans* at 22°C 48
Figure 1.11: Dauer larva compared to the other *C. elegans*.. 49
Figure 1.12: IIS pathway in *C. elegans* 52
Figure 1.13: Regulation of *ftn-1* in high and low iron conditions 58
Figure 1.14: From DNA to chromosome 66
Figure 1.15: Acetylation and deacetylation of histones 69
Figure 1.16: Stoichiometry of the NAD^+ dependent Sir2/SIRT1.. 70
Figure 1.17: The chemical structure of the polyphenol resveratrol 80

Chapter 2

Figures

Figure 2.1: Microinjection of the *C. elegans* gonad 100
Figure 2.2: Two-point mapping 104
Figure 2.3: Three-point mapping with *m* to the left or right 106
Figure 2.4: Map units convention 107
Figure 2.5: Three-point mapping with *m* in the middle 108
Figure 2.6: Map position calculations 109
Figure 2.7: EPR spectra of wildtype worms 118

Tables

Table 2.1: PCR reaction 98
Table 2.2: RT-PCR reaction 112
Table 2.3: Components for Western blot gels 113

Chapter 3

Figures

Figure 3.1: Effect of *ftn-1* RNAi on life span in *daf-2* mutants 124
Figure 3.2: Effect of reduced *ftn-1* expression on peroxide.. 128
Figure 3.3: Effect of iron supplementation on free iron level in the worm 133
Figure 3.4: GFP expression of *Pftn-1::GFP* after iron supplementation 136
Figure 3.5: Iron supplementation increases oxidative damage.. 139
Figure 3.6: Iron supplementation increases peroxide toxicity.. 141
Figure 3.7: Effect of increased iron levels on *C. elegans* life span 145
Figure 3.8: Effect of 9 mM FAC on *C. elegans* life span and.. 149
Figure 3.9: Effect of deferoxamine on free iron levels in the worms 153
Figure 3.10: Expression of *Pftn-1::GFP* on 100 µM deferoxamine 155
Figure 3.11: Effect of deferoxamine on peroxide toxicity in *C. elegans* 157
Figure 3.12: Effect of deferoxamine on protein oxidation levels.. 160
Figure 3.13: Effect of deferoxamine on life span in *C. elegans* 162

Tables

Table 3.1: Effect of *ftn-1* RNAi on life span in *daf-2* mutants 125
Table 3.2: Effect of reduced *ftn-1* expression on peroxide.. 129
Table 3.3: Effect of iron supplementation on free iron level in the worm 134
Table 3.4: Iron supplementation increases peroxide toxicity.. 142
Table 3.5: Effect of increased iron levels on *C. elegans* life span 146
Table 3.6: Effect of 9 mM FAC on *C. elegans* life span and.. 150
Table 3.7: Effect of deferoxamine on free iron levels in the worms 154
Table 3.8: Effect of deferoxamine on peroxide toxicity in *C. elegans* 158
Table 3.9: Effect of deferoxamine on life span in *C. elegans* 163

Chapter 4

Figures

Figure 4.1: Expression pattern of the *coel::GFP* plasmid in.. 172
Figure 4.2: *ftn-1* mRNA levels in the four transgenic lines 174
Figure 4.3: *ftn-1* mRNA levels of the transgenic line GA904 *(wuEx187)* 176
Figure 4.4: Brood size measurement of the *ftn-1* OE and controls 178
Figure 4.5: Effect of *ftn-1* OE on free iron levels in the worms 180
Figure 4.6: Effect of *ftn-1* OE on protein oxidation levels in *C. elegans* 183
Figure 4.7: Effect of *ftn-1* OE on protein oxidation levels in.. 186
Figure 4.8: Effect of *ftn-1* OE on survival under iron stress 188
Figure 4.9: Effect of *ftn-1* OE on peroxide toxicity in *C. elegans* 191
Figure 4.10: Effect of *ftn-1* OE on life span in *C. elegans* 195

Tables

Table 4.1: Effect of *ftn-1* OE on free iron levels in the worms 181
Table 4.2: Effect of *ftn-1* OE on survival under iron stress 189
Table 4.3: Effect of *ftn-1* OE on peroxide toxicity in *C. elegans* 192
Table 4.4: Effect of *ftn-1* OE on life span in *C. elegans* 196

Chapter 5

Figures

Figure 5.1: Life span effect of *sir-2.1* over-expression in LG100 208
Figure 5.2: Effect of out-crossing on life span in LG100 212
Figure 5.3: *sir-2.1* mRNA and protein levels after backcrossing 216
Figure 5.4: Effect of reduced *daf-2* expression on life span in GA468 218
Figure 5.5: Effect of *sir-2.1* RNAi on life span in LG100 220
Figure 5.6: LG100 but not GA468 has a dye-filling (Dyf) defect 224
Figure 5.7: SIR-2.1 levels of Dyf, non-Rol and Rol, non-Dyf lines 226
Figure 5.8: Dyf, non-Rol lines but not Rol, non-Dyf lines have a.. 228
Figure 5.9: Longevity of *C. elegans* LG100 strain segregates with.. 229
Figure 5.10: Two-point mapping of the Dyf-? mutation 233
Figure 5.11: Three-point mapping of the Dyf-? mutation I 235
Figure 5.12: Possible locations of *dyf-?(wu250)* and.. 236
Figure 5.13: Probable location of *dyf-?(wu250)* 237
Figure 5.14: Three-point mapping of the Dyf-? mutation II 238
Figure 5.15: Possible locations of *dyf-?(wu250)* and.. 239
Figure 5.16: Map position of *dyf-1(mn335)* 240
Figure 5.17: Genetic map for some of the duplication strains.. 243
Figure 5.18: Published life span of the *mDp4* strain DR1786 244
Figure 5.19: Life span of the *mDp4* duplication strain 245
Figure 5.20: Effect of *sir-2.1* on life span of DR1786 248

Tables

Table 5.1: Life span effect of *sir-2.1* over-expression in LG100 209
Table 5.2: Effect of out-crossing on life span in LG100 213
Table 5.3: Effect of reduced *daf-2* expression on life span in GA468 218
Table 5.4: Effect of *sir-2.1* RNAi on life span in LG100 221
Table 5.5: Longevity of *C. elegans* LG100 strain segregates with.. 230
Table 5.6: Life span of the *mDp4* duplication strain 246
Table 5.7: Effect of *sir-2.1* on life span of DR1786 249

Chapter 6

Figures

Figure 6.1: Life span effect of *sir-2.1* over-expression in NL3909 260
Figure 6.2: The out-crossing scheme of NL3909 263
Figure 6.3: Effect of out-crossing on life span in NL3909 265
Figure 6.4: Effect of reduced *daf-2* expression on life span in GA905 268
Figure 6.5: Previously reported *sir-2.1* RNAi with NL3909 270
Figure 6.6: Effect of *sir-2.1* RNAi on life span in NL3909 271
Figure 6.7: Life span of NL3909 background strains 278

Tables

Table 6.1: Life span effect of *sir-2.1* over-expression in NL3909 261
Table 6.2: Effect of out-crossing on life span in NL3909 266
Table 6.3: Effect of reduced *daf-2* expression on life span in GA905 269
Table 6.4: Effect of *sir-2.1* RNAi on life span in NL3909 272
Table 6.5: Hid assay of NL3909 and NL3908 and their.. 276
Table 6.6: Life span of NL3909 background strains 279

Abbreviations

AADPR	O-acetyl ADP ribose
ADPR	ADP-ribose group
ARS	Autonomously replicating sequence
ART	ADP-ribosyl transferase
C. elegans	*Caenorhabditis elegans*
CLS	Chronological life span
D. melanogaster	*Drosophila melanogaster*
D-NAAM	Drosophila nicotinamide amidase
Daf	Abnormal dauer formation
DNPH	
DR	Dietary restriction
ds RNA	double stranded RNA
Dyf	Dye filling
EPR	Electron paramagnetic resonance spectroscopy
ERCs	extrachromosomal rDNA circles
FAC	ferric ammonium citrate
Fe	Iron
FOB1	Fork block protein 1
FU	Fluorescence Units
FUdR	5-fluoro-2'-deoxyuridine
GSK	GlaxoSmithKline
HAT	Histone acetyltransferase
HIF	Hypoxia inducing factor
HDAC	Histone Deacetylase

HSP	Heat shock protein
IIS	Insulin/IGF-1 pathway
LS	Life span
NAD	Nicotine adenine dinucleotide
NGM	Nematode Growth Medium
NMU	Neuromedin U
NMURs	Neuromedin U receptors
ON	Over Night
PNC1	Pyrazinamidase/nicotinamidase 1
RISC	RNA-induced silencing complex
RLS	Replicative life span
RNAi	RNA interference
ROS	reactive oxygen species
S. cerecisiae	*Sacchamyces cerevisiae*
siRNA	small interfering RNA
S.E.M.	Standard error of the means
TBH	*tert*-butyl hydroperoxide

Chapter 1: Introduction

1.1. Ageing

Ageing usually describes the cumulative deleterious changes that occur with advancing age in organisms over time. Physiologically this includes a decline in efficiency of biological functions on molecular, cellular and tissue levels (Cummings, 2007). Typical symptoms of mammalian ageing are decreased skin elasticity, decreased mobility, decreased fertility, decreased immune response and stress resistance and increased risk of diseases, such as cancer and cardiovascular and neurodegenerative diseases. Psychological symptoms of ageing include a decline in reaction time, memory loss, and dementia. There are many theories but the biological mechanisms remain unclear.

Although most organisms age, there are some exceptions to the rule. For example, some cnidarians, such as *Hydra* or *Urticina felina,* do not show any detectable signs of ageing (Martínez, 1998; Schmich et al., 2007). Does that mean organismal ageing is avoidable?

1.2. Possible explanations for ageing

So far, the biological mechanisms that cause ageing are still unclear. However, the measurable and visible changes that occur and accumulate the older an organism gets provide the basis for a number of theories of ageing. In fact, there are over 300 theories about ageing and longevity (Medvedev, 1990). So far, the closest thing to a consensus in the scientific community about the mechanistic causes of ageing and longevity is the involvement of

molecular damage and somatic maintenance, respectively. Influential theories of ageing include the “rate of living theory of ageing” of Max Rubner and Raymond Pearl (1908, see section 1.2.2.) and the “oxidative damage theory of ageing” of Denham Harman (1956, see section 1.2.2.1.).

1.2.1. Energy metabolism and ageing

The diversity in life span that different organisms can attain is very great. For instance, a rat has a life span of three years, whereas Bowhead whales can live over 200 years (George et al., 1999). One of the earliest ageing theories trying to explain these differences in life span between species was the “rate of living theory of ageing”, which originates in work in 1908 by the physiologist Max Rubner. He noted that the differences in life span in mammals could be correlated to their metabolic rate and size. Smaller mammals have a higher metabolic rate and shorter life span, whereas larger mammals have a lower metabolic rate and longer life span (Rubner, 1883; Rubner, 1908; Speakman, 2005). Generally, the metabolic rate is defined as the rate in which the body consumes calories and is described as oxygen consumption per mass per time ($l/O_2/kg/h$). For instance, horses have a metabolic rate of 0.2 l/kg/h and life up to 35 years, whereas squirrels have a metabolic rate of 1 l/kg/h and only life up to 7 years (Rubner, 1908). It was then proposed in 1928 by Raymond Pearl, that the amount of energy metabolized per mass of an organism in the course of a full, long life is constant for most mammals (Pearl, 1928). This constant is sometimes referred to as the life energy potential. Thus, the faster the metabolic rate is,

the more rapidly the fixed allotment of energy transduction capacity expires, and the more quickly expires the organism.

However, there are a number of reasons to think that the rate of living theory is probably incorrect. For instance, the life energy potential in mammals shows striking variation, even between closely related species. For example, among rodents, rats can only attain an age of three years, whereas similar sized naked mole rats can life up to 28 years (Finch, 1990; Perez et al., 2009). Furthermore, increased metabolic rate through exercise does not shorten life span in mammals (Holloszy et al., 1985; Lee et al., 1995). Long-lived insulin-like growth factor (IGF, see section 1.3.3.) mutants of *Drosophila* do not have reduced metabolic rates (Hulbert et al., 2004) and dogs with higher metabolic rates have been reported to live longer than ones with lower rates (Speakman et al., 2003). These are just a few examples in which the metabolic rate is not connected to life span.

1.2.2. Damage theory of ageing

Many ageing theories are based on the idea that accumulated damage is the main cause of ageing. These include the “wear and tear”, “waste accumulation”, “errors and repair”, “DNA damage”, “mitochondrial damage”, “disposable soma” and “damage from metal ions” theories. The specifics of most of these theories are not important for the understanding of this thesis and are therefore discussed elsewhere (Medvedev, 1990). However, it is true that damage increases in an organism over time, and it is widely thought that this is the main cause of ageing.

It remains unclear whether molecular damage really contributes to ageing or just a side effect. For instance, errors in DNA repair contribute to DNA damage. DNA repair deficient mice have higher mutation rates but not accelerated ageing (Narayanan et al., 1997). The effect of over-expression of DNA repair genes in mice is controversial, with one study showing delayed ageing (Matheu et al., 2007), but another not seeing this effect (Zhou et al., 2001). A prominent version of the damage theory of ageing is the oxidative damage theory, which is discussed in more detailed below.

1.2.2.1. The oxidative damage theory of ageing

In 1956 Denham Harman proposed the free radical theory, which developed into the oxidative damage theory. The latter views ageing as a result of molecular damage caused by reactive oxygen species (ROS), both free radical or otherwise (Beckman and Ames, 1998; Harman, 1956). The theory is that an imbalance between prooxidant and antioxidant defenses led to an accumulation of oxidative damage, caused by ROS. This causes damage to all classes of biomolecules, including DNA, proteins and lipids. Various observations are consistent with this theory. For example, long-lived species sometimes show reduced ROS production (Beckman and Ames, 1998; de Castro et al., 2004; Herrero and Barja, 1999; Ku et al., 1993; Sohal et al., 1993). The oxidative damage theory has been linked to the “rate of living theory”, in that an increased metabolic rate might led to greater ROS production. Particularly during mitochondrial respiration and with this more damage thereby accelerating ageing (Beckman and Ames, 1998; Finkel and Holbrook, 2000).

1.2.2.2. Oxidative damage in biological systems

During normal metabolism, oxidation occurs in the form of electron transfer from an electron-rich substances, such as oxygen, to electron deficient ones, called oxidizers. In this process molecular oxygen (O_2), is converted into potentially toxic reactive oxygen species (ROS) such as $O_2\bullet^-$, H_2O_2 and •OH. ROS exhibit a high degree of reactivity, which in the case of free radical ROS is caused by their unpaired electrons (Gutteridge and Halliwell, 1989).

$O_2 + e^- + H^+ \Rightarrow HO_2\bullet$ (hydroperoxyl radical)

$HO_2\bullet \Rightarrow H^+ + O_2\bullet^-$ (superoxide radical)

$O_2\bullet^- + 2H^+ + e^- \Rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + e^- \Rightarrow OH^- + OH\bullet$ (hydroxyl radical)

$OH + e^- + H^+ \Rightarrow H_2O$

Figure 1.1: List of redox reactions showing the generation of different ROS
(Reproduced from (Flora, 2009)

Possible causes for ROS production are UV, X-ray and gamma irradiation, metal-catalyzed reactions, neutrophils and macrophages during inflammation, environmental pollutants and by products of the mitochondria-catalyzed electron transport chain reaction (Cadenas, 1989). The main *in vivo* source of ROS, such as hydrogen peroxide and superoxide anions, are the mitochondria (Boveris and Cadenas, 1975; Chance et al., 1979; Loschen et al., 1971).

In 1934, Haber postulated that the less reactive ROS, such as superoxide and hydrogen peroxide could react to produce the highly reactive hydroxyl radical (Haber, 1934). This reaction, the Haber-Weiss reaction (1), is catalyzed by metal ions and can be split into two separate reactions ((2) and (3)), which is nowadays known as the Fenton reaction.

(1) $O\bullet_2^- + H_2O_2 \Rightarrow O_2 + OH^- + OH\bullet$

(2) $Fe^{3+} + O\bullet_2^- \Rightarrow Fe^{2+} + O_2$

(3) $Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH- + OH\bullet$

Other transition metals, such as copper, can catalyze these reactions, but the Haber-Weiss and iron catalyzed Fenton reaction are now considered the main source for the highly damaging hydroxyl radical in biological systems (Barbouti et al., 2001; Fridovich, 1978; Keyer et al., 1995; Liochev, 1999; Mello-Filho and Meneghini, 1991; Meneghini, 1997).

The occurrence of oxidized DNA bases can be used as a marker for ROS-mediated DNA damage (Helbock et al., 1999). Oxidized DNA leads to genetic mutations and alterations in transcription. Lipids are constituents of all membranes in cells, and damage to lipids can compromise membranes, sometimes result in cell death. Vulnerable targets for ROS in lipids are the double bonds in polyunsaturated fatty acids. ROS can remove a hydrogen atom at these double bonds and create radical species able to react with other atoms. This can start a chain reaction, affecting ionic channels, membrane transport proteins/enzymes and ultimately destroy the lipid bilayer itself (Kehrer, 2000).

ROS generated oxidation of protein produces stable as well as reactive products (Dean et al., 1997). Reactive products include the protein hydroperoxides, which can generate further radicals by interaction with transition metals (Dean et al., 1997). Another typical side effect of protein oxidation is the carbonylation of protein side chains, such as lysine, arginine, proline and threonine. A variety of ROS can cause this kind of oxidation, but metal-catalyzed oxidation, involving the Fenton chemistry and hydroxyl radical formation is the most likely usurpator (Stadtman, 1990). Not all inactive oxidized proteins are removed, therefore oxidized proteins accumulate over the years and are correlated with age associated damage and disease, such as diabetes, atherosclerosis and neurodegenerative diseases (Greeve et al., 2004; Kehrer, 2000; Stadtman, 1990; Yang et al., 2005). In my studies I used protein carbonyl measurements to determine the level of oxidative damage in *C. elegans* (see Chapter 3 and 4).

1.2.2.3. Heavy metals causing oxidative damage

Heavy metals play a fundamental role in many biological processes, but can be harmful at high concentrations. Numerous studies have identified several metals as toxic and carcinogenic. For instance, iron, cadmium, chromium, mercury, arsenic and led can act as strong oxidizers due to their free d-orbitals. Several studies have shown their ability to catalyze ROS production, leding to oxidative damage in proteins (Kagi and Schaffer, 1988; Samuni et al., 1981), lipids, DNA and RNA (Hartwig, 1995; Kalia and Flora, 2005; Tsang et al., 1996).

There are numerous reports about deleterious effects of metals in biological systems. For instance, high concentrations of copper can cause proliferation of cancer cells (Brewer et al., 2000) and ROS generated by cobalt (II) complexes can cause toxicity in the heart (Bucher et al., 1999; Leonard et al., 1998). Arsenic causes oxidative stress in mammalian and human cells (Applegate et al., 1991) and in laboratory animals (Chou et al., 2005; Liu et al., 2001). Led is reported to cause oxidative stress via increased ROS production, resulting in weakened antioxidant defense systems in cells (Flora, 2002; Lawton and Donaldson, 1991; Sandhir and Gill, 1995). A similar effect results from the inhibition of superoxide dismutase by led (Tripathi et al., 2001) and the sensitivity of catalase to led toxicity (Sandhir and Gill, 1995). Furthermore, iron overload increases the risk of various diseases, such as vascular disease, cancer and certain neurological conditions (Berg et al., 2001; Siah et al., 2005).

Therefore effective maintenance of metal homeostasis is essential for health. To keep this balance, organisms have developed several ways to regulate levels of heavy metals, such as membrane-bound metal transporters, metal-chaperones and metal storage proteins (Cai et al., 2005; Harrison et al., 2000; Nelson, 1999).

Henceforth I will mainly focus on iron homeostasis and iron-generated oxidative damage, since this is major topic of my thesis (see Chapter 3 and 4).

1.2.2.4. The role of iron in oxidative damage

Iron plays a central role in many essential cellular processes such as oxygen transport, heme synthesis, electron transport, neurotransmitter synthesis, xenobiotic detoxification, mitochondrial energy metabolism and DNA synthesis. Its oxidized and reduced forms are referred to as ferric (III) and ferrous (II) iron.

All cells contain a “low molecular mass” iron pool, which means a free iron pool, potentially catalytic for free-radical reactions. Free iron or excessively high concentrations of it are toxic due to its ability to generate oxidative stress by catalyzing the Fenton reaction. Here iron (II) is oxidized by H_2O_2 to iron (III) producing the damaging oxygen radical OH• (Gutteridge and Halliwell, 2000). Oxidative stress interferes with iron metabolism in cells and tissues. Increased generation of $O_2•^-$ and H_2O_2 can create conditions favorable for OH• formation. For example, $O_2•^-$ can release iron ions from ferritin, while H_2O_2 displaces iron from Fe-S proteins. Hence ROS, in the form of the highly reactive OH•, is produced whenever iron is available for the Fenton reaction. This is, for example, important in mitochondria, where normal cellular respiration provides the substrates, in the presence of iron, for OH• generation.

1.2.2.5. Defense mechanisms to oxidative damage in biological systems

The cellular production of ROS is balanced by antioxidant defenses (Halliwell, 1996). Generally, antioxidants prevent or limit oxidative stress by

counteracting or neutralizing ROS. Antioxidants may be divided into enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase (GP_X). Examples for non-enzymatic ones are vitamin C/E, carotenoids, thiol antioxidants, flavonoids, melatonin and ferritins. Some are described further below. Here I will focus principally on enzymatic antioxidants and ferritins.

1.2.2.5.1. Enzymatic antioxidants

A well-characterized enzymatic antioxidant is catalase. It is present in organisms ranging from aerobic bacteria to plants and animals (Mates et al., 1999) and is usually located in the peroxisomes. It catalyzes the reaction of hydrogen peroxide to water and oxygen.

$$2\ H_2O_2 \Rightarrow 2\ H_2O + O_2$$

Glutathione peroxidase (GPx) is another important antioxidant. In the presence of tripeptide glutathione (GSH) GPx can donate two electrons to reduce peroxides (Mates et al., 1999). GPx catalyzes peroxides to water or alcohol, while oxidizing GSH. This decreases the available peroxide for the Fenton reaction.

$$2GSH + H_2O_2 \Rightarrow GS\text{-}SG + 2\ H_2O$$

Another well-known enzymatic antioxidant is superoxide dismutase (SOD). Eukaryotic organisms have three kinds of SOD: cytosolic CuZn SOD,

extracellular CuZn SOD and mitochondrial Mn SOD (Fridovich, 1995). SOD catalyzes the reaction of superoxide ion to hydrogen peroxide (Desideri and Falconi, 2003; McCord and Fridovich, 1969).

$$O_2\bullet^- + 2H^+ \Rightarrow H_2O_2$$

1.2.2.5.2. Ferritins in vertebrates

Ferritins and metallothioneins (MTs) are major metal regulating proteins. Hemoglobin, transferin and ferritin are responsible for controlling the amount of available reactive iron in organisms (Halliwell and Gutteridge, 1986). Ferritin is the major iron storage protein in prokaryotes and eukaryotes regulating cytosolic concentration of iron by storing excess iron and keeping it away from the nucleus and other organelles. Ferritin genes are present in most organisms, except for yeast, which uses alternative mechanisms to store excess iron. Although ferritins from different species may have largely different sequences, with protein sequence identities as low as 15%, their three-dimensional structures are highly conserved (Levi et al., 2002).

The structure of ferritin proteins is heteropolymeric, consisting of 24 protein subunits. The subunits consist of two types, the light (L) and heavy (H) type subunits with molecular weight ranging from 19-21 kDa. They build a shell with a cavity in the middle that can store up to 4500 iron atoms. Within this cavity, the H-subunits of ferritin oxidize Fe (II) to Fe (III).

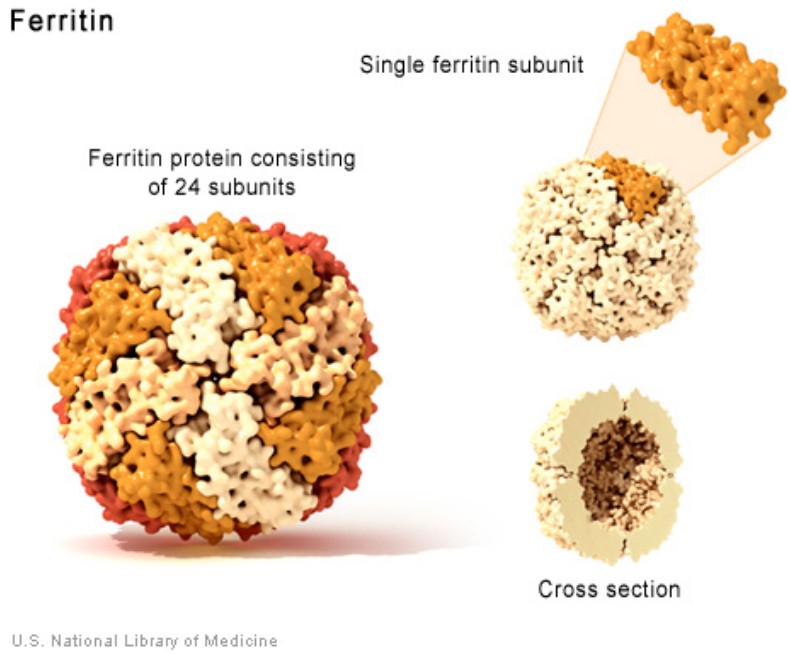
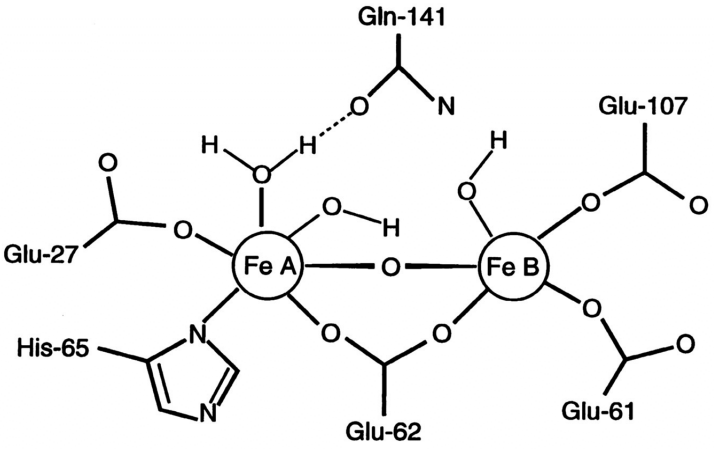


Figure 1.2: Image of a ferritin protein with its different subunits (Reproduced from Genetics Home Reference ghr.nlm.nih.gov/ U.S. National Library of Medicine).

1.2.2.5.2.1. Ferritins as antioxidants

The ability of ferritins to sequester large amounts of Fe (II) rendering it unavailable for Fenton chemistry is defines this iron storage protein as an effective antioxidant (Levi and Arosio, 2004). Besides preventing the hydroxyl radical production, ferritins also use O_2 and H_2O_2 as oxidants in the iron binding reaction catalyzed in the ferroxidase center (Bou-Abdallah et al., 2002; Su et al., 2005; Zhao et al., 2003). The reaction with iron begins with the binding of Fe (II) to the ferroxidase centre. Ferroxidases are enzymes that catalyze the conversion of ferrous iron to ferric iron. Iron interacts with oxygen, is oxidized to Fe (III) and then migrates to the cavity where it nucleates and aggregates to form the iron core. Here also H_2O_2 is produced that serves for further oxidation reactions of Fe (II) (see Figure 1.3)(Arosio and Levi, 2002; Zhao et al., 2006). Only the H-chain subunits have a

ferroxidase activity, whereas the L-chain subunits are responsible for the iron transfer from the ferroxidase center to the iron core (Santambrogio et al., 1993). Considering its capacity to reduce ROS, it is not surprising that ferritin respond to oxidative stress by increased expression (Cozzi et al., 2004; Thompson et al., 2002; Torti and Torti, 2002).



Ferritin ferroxidation

$$2\ Fe\ (II) + O_2 + 4\ H_2O \Rightarrow 2\ Fe\ (O)OH_{(core)} + H_2O_2 + 4\ H^+ \quad (1)$$

$$2\ Fe\ (II) + H_2O_2 + 2\ H_2O \Rightarrow 2\ Fe\ (O)OH_{(core)} + 4\ H^+ \quad (2)$$

Fenton reaction

$$Fe\ (II) + H_2O_2 \Rightarrow Fe\ (III) + OH^- + OH\bullet \quad (3)$$

Figure 1.3: Ferroxidase reaction of ferritins. The ferroxidase center can use O_2 (1) or H_2O_2 to generate the iron core (Reproduced from (Arosio and Levi, 2002).

Ferritin protects cells against UV induced DNA breaks and iron or oxidative agents induced oxidative damage (Cai et al., 1998; Campanella et al., 2009; Levi and Arosio, 2004; Thompson et al., 2002). Furthermore, over-expression of H-chain ferritin in cells decreases cellular iron and ROS production and increases resistance to oxidative stress (Cozzi et al., 2000;

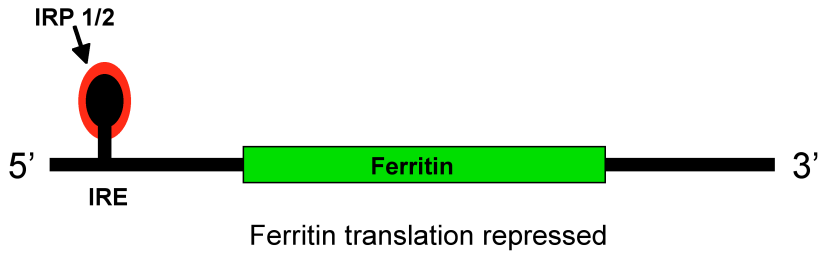
Epsztejn et al., 1999; Linsenmayer et al., 2005; MacKenzie et al., 2008; Yuan et al., 2004). Reduced ferritin levels had the opposite effect (Cozzi et al., 2004). The bacterial ferritins can protect against peroxide stress and iron-induced oxidative damage (Andrews et al., 2003; Chiancone et al., 2004). In *Drosophila*, over-expression of mitochondrial ferritin increased their resistance to the oxidizing agent paraquat (Missirlis et al., 2006).

1.2.2.5.2.2. Ferritin regulation in vertebrates

Induction of ferritin gene expression by iron is regulated by transcriptional and post-transcriptional mechanisms that vary between organisms (Hintze and Theil, 2006; Nichol et al., 2002; Torti and Torti, 2002).

In vertebrates, expression of ferritin is regulated at the translational level through cytosolic proteins called iron-regulatory protein 1 and 2 (IRP1/2). In the 5'- or 3' untranslated regions of the H and L-ferritin mRNAs are iron-responsive elements (IREs) (Wallander et al., 2006). The IRP1/2 inhibit ferritin gene expression by binding to IREs. When iron levels are low, these cytosolic proteins bind to ferritin 5' IREs and suppress ferritin translation. In this way iron sequestration is decreased, thereby increasing iron levels available. In the case of high iron levels, IRPs detach from the IREs, which leds to activation of ferritin translation and increased iron sequestration.

Low iron: active IRP1 and IRP2



High iron: converted IRP1 and degraded IRP2

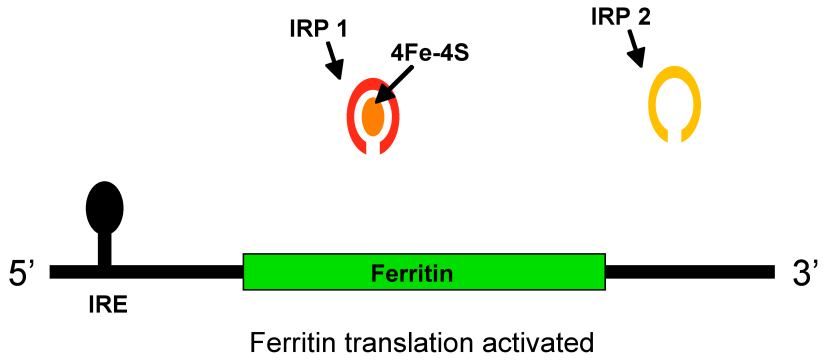


Figure 1.4: Ferritin regulation in vertebrates. Translation of ferritin mRNA is regulated by binding of iron response protein (IRP) to iron response elements (IREs) located in either 5'- or 3' untranslated regions of the ferritin mRNA. During low iron conditions, IRP1 and IRP2 bind to 5' and 3' IREs, resulting in translational repression. During high iron conditions, IRPs lose their binding to IREs, increasing translation (Reproduced from (Torti and Torti, 2002).

Iron also induces the conversion of IRP1 from a RNA-binding protein to a cytosolic aconitase without RNA-binding ability, decreasing the affinity to the IRE. This change is caused by a [4Fe-4S] cluster formation. In addition, the degradation of IRP2 is supported by iron (Guo et al., 1995; Iwai et al., 1998). IRP activities/levels can also be affected by various forms of oxidative stress (Ponka, 1999). This involves antioxidant responsive elements (ARE) upstream of the ferritin promoters (Hintze and Theil, 2005; Iwasaki et al., 2006; Torti and Torti, 2002).

1.2.2.6. Iron chelators

Another way of inhibiting iron-induced ROS production, besides using ferritins, is the chelation of iron. During the chelation process an iron complex is formed in which the iron ion carries a charged or uncharged electron donor that acts as ligand. This ligand can be in a monodenate, bidenate or multidenate state, describing the chelators ability to attach or co-ordinate to one, two or more donor atoms. Pre-incubating cells with iron-chelating agents or H-ferritins can sequester iron and decrease oxidative damage (Balla et al., 1992). In this thesis I investigate whether iron-generated molecular damage and iron homeostasis are determinants of ageing rate in *C. elegans*. Next, I will describe more the nascent field of biogerontology and the important role played in it by *C. elegans*.

1.3. Mechanisms of ageing

The aim of biogerontology is to understand the biological basis of ageing. A powerful approach within biogerontology is to identify interventions that slow ageing, and then investigate the mechanisms by which such interventions act.

1.3.1. Dietary Restriction (DR)

DR is an intervention involving reduction of nutrients without causing malnutrition. DR is one of the most widely studied interventions that increase life span. Typical characteristics of DR in mammals are reduction of metabolic rate (body temperature, body fat, blood glucose levels, growth factors),

reproduction and generation of mitochondrial ROS, while physical activity is increased in higher age compared to controls (Holehan and Merry, 1985a; Holehan and Merry, 1985b; Holloszy and Schechtman, 1991; McCarter et al., 1997; Weed et al., 1997; Weindruch and Walford, 1988). Organisms under DR also show increased resistance to heat and oxidative stress (Heydari et al., 1993; Sohal and Weindruch, 1996). In addition, DR reduces age-related disorders, such as cardiovascular disease, diabetes and cancer (Youngman et al., 1992).

The effects of DR on life span were first described in 1935 in rats (McCay et al., 1935). Since then many studies have confirmed this effect, and also seen it in yeast, *Drosophila* and *C. elegans* (Lane et al., 2001; Lin et al., 2002; McCay et al., 1935; Partridge et al., 2005; Piper and Partridge, 2007; Walker et al., 2005; Weindruch and Walford, 1988; Weindruch et al., 1986).

How DR affects longevity in these model organisms is unclear. Some studies suggested an involvement of reduced metabolic rate, in agreement with the rate of living theory (see section 1.2.1.), insulin/IGF-1 signaling (see section 1.3.3.) (Bordone and Guarente, 2005; Ramsey et al., 2000; Walker et al., 2005; Weindruch and Walford, 1988), or the sirtuins class III histone deacetylases, (see section 1.6), but these findings are challenged by other studies (Bartke, 2001; Clancy et al., 2002; Houthoofd et al., 2003; Huang et al., 2004; Lakowski and Hekimi, 1998; McCarter et al., 1985; Speakman, 2005).

Another hypothesis suggested that DR treated organisms live longer because less ROS are produced, linking the DR effects to the oxidative damage theory of ageing (Sohal and Weindruch, 1996; Weindruch, 1996)

(see section 1.2.2.1.). DR is reported to improve health in primates (rhesus monkeys (Anderson and Weindruch, 2006; Lefevre et al., 2009), which leds to a reduction in age-related death rate in monkeys (Colman et al., 2009). The effect of DR on human life span is still unclear.

It should also be noted that DR effects on life span are not always seen. Recent studies have shown that DR effects on life span in mice are strain-dependent. For instance, the mouse strain dilute brown non-agouti (DBA) mice did not show an increased life span upon DR (Forster et al., 2003), and neither did some inbred strains (Liao et al., 2010) or wild mice (Harper et al., 2006).

1.3.2. Single-gene effect on life span

Another important part of biogerontology is the study of single-gene effects on life span. In 1988, a single gene mutation, previously isolated by a screen (Klass, 1983) was found to also extended life span of *C. elegans* (Friedman and Johnson, 1988). Today, single gene-effects on life span are widely studied and some are known to be involved in the insulin/insulin-like growth factor signaling (IIS) pathway (Kenyon et al., 1993). The effect of reduced IIS on life span is widely conserved in the animal kingdom, since inhibition of IIS has been shown to increase life span in *C. elegans*, *Drosophila* and mice (Brown-Borg et al., 1996; Kenyon et al., 1993). Over-expression as well as mutation of genes can also increase life span. For example, heat shock proteins can increase life span in *C. elegans* and *Drosophila* (Swindell, 2009), although in at least one case over-expression of a heat shock gene in mice caused a decrease in life span (Morrow et al.,

2010; Vanhooren et al., 2008). Single-gene manipulations, such as knockdown or over-expression, are nowadays common methods to analyze the function of single genes, including their effect on life span. In this thesis I mainly investigate effects on *C. elegans* life span of two genes, *ftn-1* and *sir-2.1*. Details are presented and discussed in depth in the result chapters.

1.3.3. The insulin IGF-1 signaling (IIS) pathway and ageing

As described already, the IIS pathway is a “hot topic” in biogerontology. Generally, the hormone insulin is a regulator of carbohydrate and fat metabolism. Reduced production or resistance to insulin can cause diabetes mellitus. It is therefore surprising that mutations in this important pathway produce life span increasing effects in species ranging from *C. elegans* to mice, and perhaps even to humans (Bartke, 2008; Bluher et al., 2003; Clancy et al., 2001; Holzenberger et al., 2003; Kenyon et al., 1993; Kenyon, 2010; Kimura et al., 1997; Longo, 2003; Riddle et al., 1997; Tatar et al., 2003; Tu et al., 2002). This pathway was first connected to life span in the model organism *C. elegans.* Additionally, IIS mutants show increased resistance to UV radiation and oxidative and thermal stress (Fabrizio et al., 2003; Holzenberger et al., 2003; Kops et al., 2002; Larsen, 1993; Lithgow et al., 1995; Longo, 2003; Murakami and Johnson, 1996; Nemoto, 2002; Tran et al., 2002).

This paradox of a deleterious effect (diabetes) on the one hand and a beneficial one (increased stress resistance and life span) on the other hand is so far unsolved. Some studies imply that the effect of IIS on ageing is tissue specific. For instance, mutations in the insulin receptor in adipose tissue (fat

tissue) cause life span extension in mice, whereas in the liver it results in diabetes (Bluher et al., 2003; Michael et al., 2000). In invertebrates, specifically nematodes and fruit flies, the life span extending effect of IIS mutations is also associated with adipose tissue. The fat body in the fly and the intestine in the worm, both of which serve as adipose tissue, among other functions (Giannakou et al., 2004; Hwangbo et al., 2004; Libina et al., 2003). This link to fat tissue and longevity in model organisms is especially interesting given that variation in genes responsible for fat transport in humans is also connected to longevity (Barzilai et al., 2003; Geesaman et al., 2003).

1.4. Model organisms in ageing research

Model organisms in ageing research are particularly useful for generating and testing hypotheses out and obtaining a deeper insight into the mechanism of ageing in humans. It is a lot easier to standardize genetic background, and environmental and food effects in animals kept in controlled laboratory conditions than in humans. Additionally, genetic manipulation like single-gene changes, can give a deeper insight into certain biological processes and pathways and their involvement in ageing. Furthermore, the shorter life span in model organisms allows a quicker progress in research than studies in humans would ever provide.

In this thesis I used the nematode *C. elegans* as model organism, therefore I will mainly concentrate on this invertebrate. However, other important model organisms in ageing research include the budding yeast

Saccharomyces cerevisiae, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus*.

1.4.1. *C. elegans* as a model organism for studying ageing

Caenorhabditis elegans is a small, free-living soil nematode. In nature, it feeds on bacteria and microbes, particularly that develop on compost. In 1948, *C. elegans* was first suggested as a multi-cellular organism to study genetic effects on physiology (Dougherty and Calhoun, 1948). In 1974, Sydney Brenner, the godfather of *C. elegans* research, introduced this roundworm as a model organism for genetic studies (Brenner, 1974).

This nematode is used as a model organism for many reasons, such as economy, easy maintenance in the laboratory, easy observation and manipulation. Its small size of only 1 mm makes it possible to grow large numbers in mass culture. The typical food source used in labs is the *E. coli* strain OP50 (Brenner, 1974). Since they are largely transparent throughout their life span, the observations of phenotypical changes or gene expression studies are easy. Manipulations of single genes are typically done by using chemical mutagenesis, knock down via RNA – mediated interference (RNAi) or creating transgenic lines over-expressing the chosen gene.

In *C. elegans* the whole cell lineage has been described (Sulston et al., 1983) and its genome is fully sequenced. *C. elegans* mutant strains are distributed via the Caenorhabditis Genetics Center (CGC). Furthermore, it is easy to test transgenes or mutations in various mutant backgrounds by genetic crossing. Long-term storage is possible at -70°C, which is particularly

convenient. Another big advantage of this model organism, especially in biogerontology, is its short wildtype life span of 18-20 days at 20°C.

1.4.2. *C. elegans* genetic terminology

C. elegans gene names are usually chosen according to the mutant phenotype of the gene concerned, for instance Unc describes an uncoordinated (abnormal) movement phenotype. *C. elegans* gene names are three letter abbreviations, such as *unc*, followed by a hyphen and a number. The number describes the order in which this gene was discovered. For example, *unc-31* was the 31st *unc* gene discovered in *C. elegans*. All gene names are written in italics. Proteins are written in capital letters and not in italics. For instance, the protein encoded by *unc-31* is UNC-31. If a phenotype is described, the first letter are written capital and, again, no italics is used. Therefore phenotype of *unc-31* is Unc.

In case of a mutation in a gene, the same conventions apply as described above, but the allele name is added. This consists of one or two letters, the lab-code designated to each *C. elegans* lab, followed by a number that describes the order in which the alleles were described by this lab. For instance, *wu* is the designated code for the Gems lab and the number n would describe that this allele is the nth mutation described in this lab.

The convention for naming transgenic strain is similar. First comes the lab code, then the specification of whether the transgene array is integrated (*Is*) or extrachromosomal (*Ex*), followed by the description of the transgene array in brackets (Horvitz et al., 1979). See Figure 1.5 for a detailed example.

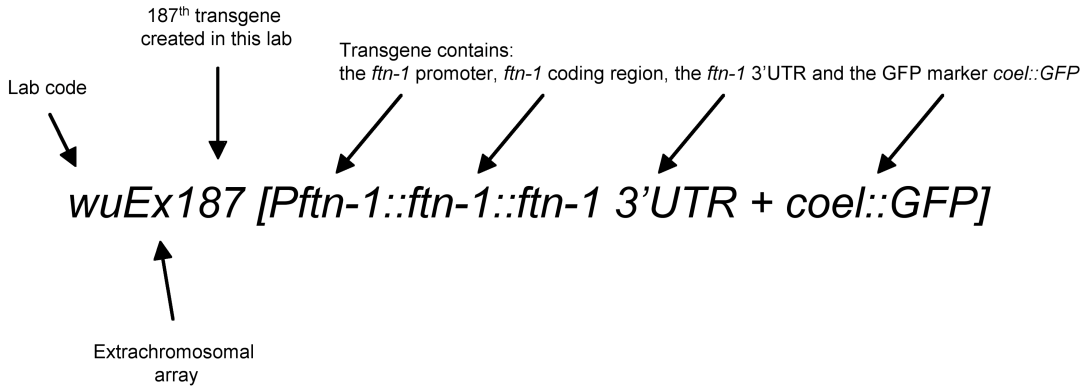


Figure 1.5: Naming convention for the transgenic *C. elegans* strain GA904. Example of the *ftn-1* over-expresser line described in Chapter 4.

Strain names are given by combining the strain lab code (GA for Gems lab) and the number allocated to the scientist generating the strain. My number is 900. Every further strain I designed added to this number in chronological order. For instance, the first strain I designed was named GA900, the next GA901 etc.

1.4.3. Common *C. elegans* mutant classes

Mutations in genes can result in phenotypic changes. Since I used different mutant phenotypes throughout this thesis, I will explain a few of the more common mutant classes here.

rol Loss of function of *rol* genes cause the worms to rol in circles, showing a horseshoe shaped form. Many *rol* genes encode a cuticle collagen responsible for normal cuticle formation.

unc The Unc phenotype describes an uncoordinated movement. Different mutations of unc genes cause different types of movement abnormalities, such as coiling, kinking and failure to move forward or backwards, or complete paralysis.

dpy Dumpy are shorter and fatter than wildtype worms. This phenotype can range from slightly fat to severely fat and short body forms.

lon Lon (long) mutants are abnormal in pathways regulating worm body length. *lon* mutant worms are up to 50% longer than wildtype.

bli *bli* (blister) genes are required for proper adhesion of the cuticle in adult worms. Bli mutants show blister formation on their cuticle, visible as large bubbles on the surface of the worms.

egl This phenotype describes an egg-laying defect. Here, mutations cause the worms to retain the eggs, resulting in bagging. Here the eggs hatch internally and the resulting progeny eat their mother from within.

1.4.4. *C. elegans* anatomy

Typical of nematodes, *C. elegans* worms have a round cylindrical body essentially consisting of an outer tube (body wall) and an inner tube. The body wall is made of cuticle, hypodermis, excretory system, neurons and muscles. The inner tube consists of the pharynx, the intestine and the gonad.

The pharynx is located in the anterior part, the head region, which is responsible for up-take of food, such as bacteria. It is directly connected to the intestine, which ends in the posterior part in the anus, where defecation occurs. Differences between hermaphrodites and males lie mainly in their reproductive system. The reproductive system consists mainly of the gonad, where the gametes are produced.

1.4.4.1. *C. elegans* hermaphrodites

In hermaphrodites, the gonad consists of a somatic gonad, the germ line and the egg-laying apparatus. Two distal arms of the gonad (ovaries) converge onto a central uterus via two spermathecae. In the spermathecae, oocytes are fertilized by the sperm as they pass through. The eggs pass along the uterus and are then released through the vulva, which penetrates the body wall in the ventral midsection (see Figure 1.6) (Brenner, 1973; Lewis and Fleming, 1995; Sulston, 1976).

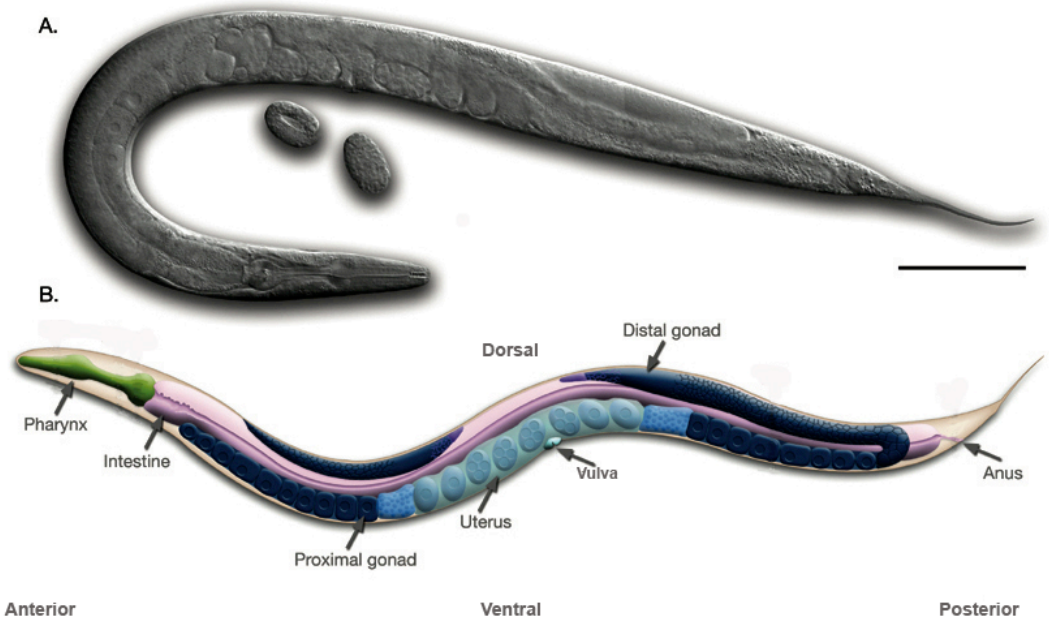


Figure 1.6: *C. elegans* hermaphrodite anatomy. Both images represent an adult hermaphrodite. **A**. View using Nomarski microscopy. **B.** Shows a schematic drawing of *C. elegans* (Reproduced from Wormatlas.org et al.). The scale bar shows 0.1 mm

1.4.4.2. *C. elegans* males

The rare occurring males of *C. elegans* show certain difference in body morphology to the hermaphrodite worms, particularly at their posterior end. These changes begin in the L2 stage, visible as a slight thickening of the tail (see Figure 1.7, E.) (Sulston et al., 1980; Sulston and Horvitz, 1977). Here the sexual organ develops into a copulatory apparatus with 9 sensory rays that help to transfer the sperm through the vulva of the hermaphrodites (Liu and Sternberg, 1995). Furthermore, their reproductive system has only a single gonad arm. This includes a somatic gonad and germline that is connected to the proctodeum and anus through the seminal vesicle and vas deferens (see Figure 1.7).

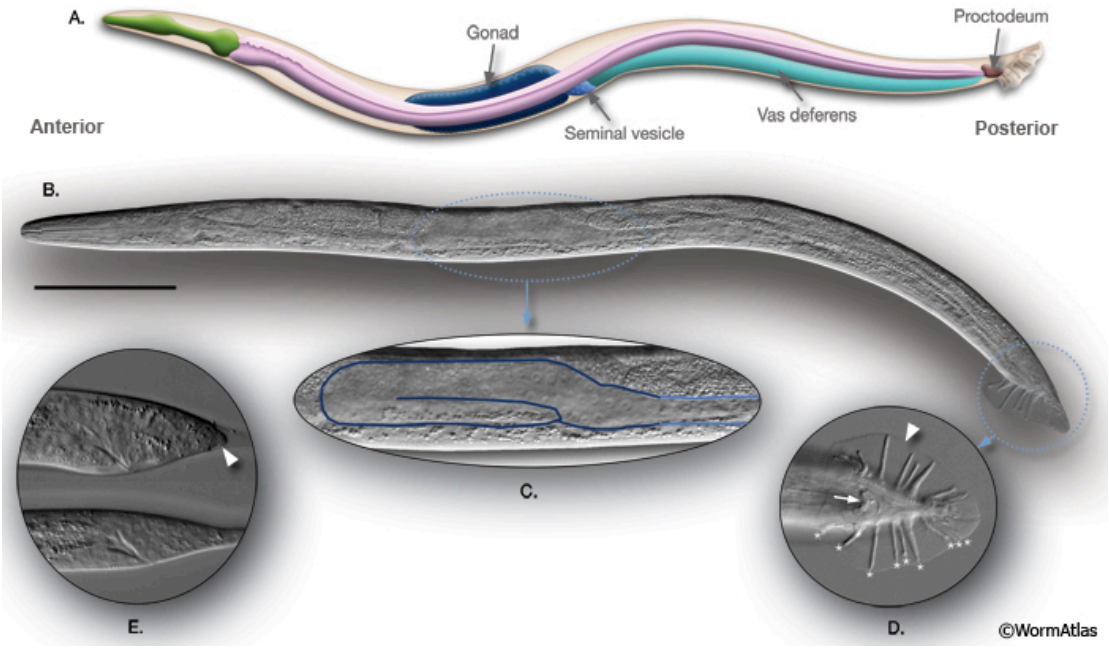


Figure 1.7: *C. elegans* male anatomy. A. Schematic figure of male anatomy. **B.** View using Nomarski microscopy, scale bar shows 0.1 mm. **C.** Male gonad. **D.** Male tail enlarged. **E.** Male tail in L3 staged larva (Reproduced from Wormatlas.org et al.).

1.4.4.3. *C. elegans* nervous system

The *C. elegans* nervous system consists of 302 neurons in total, with 118 different neuron classes. The most basic classification separates them to sensory, inter- and motor-neurons. The sensory neurons gather and transfer sensory stimuli from the environment, interneurons receive and transfer information to other neurons and motor neurons transmit information to the muscle cells. The distribution of the nerve cells is mainly in the head, tail and along the ventral cord and build the somatic nervous system (Ward et al., 1975; White et al., 1986) (Figure 1.8).

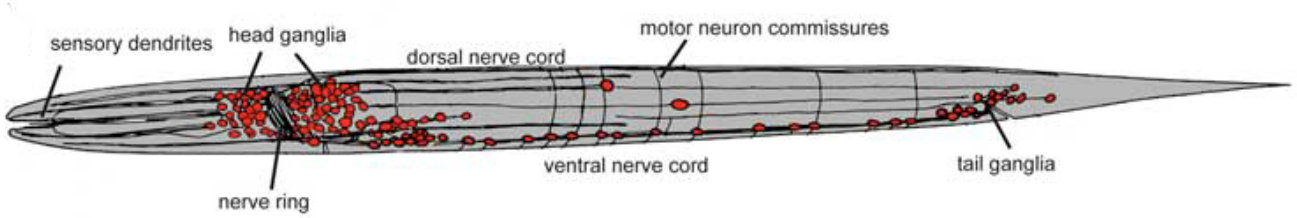


Figure 1.8: Nervous system of *C. elegans* (Reproduced from (White et al., 1986).

Since the highest concentration of neurons is in the head area, this is occasionally referred to as the *C. elegans* “brain”. The “brain” consists mainly of the cell bodies of sensory neurons that are organized in ganglia around the pharynx. However, the pharynx has its own autonomous nervous system, consisting of 20 neurons that create the second so-called pharyngeal nervous system (White et al., 1986). Through the nerve ring, a horseshoe-shaped group of axons, the sensory neurons connect to the interneurons and transfer their information about chemical, mechanical and thermal stimuli of their surroundings. The interneuron axons connect to the ventral cord and from

there to the motoneurons, allowing the worm to react to external stimuli and conditions. The nervous system of the *C. elegans* males has in total 473 neurons, is therefore bigger than the one of the hermaphrodites (79 neurons and 36 support cells more) (Sulston et al., 1980).

1.4.4.3.1. Sensory neurons

Movement and behavior of *C. elegans* are determined by chemotaxis, thermotaxis, aerotaxis and mechanotaxis. The term –taxis describes a response to chemicals (chemo-), temperature (thermo-), air (aero-) and mechanic (mechano-) resulting in a movement away or towards the harmful/attractant substances. Stimuli inducing those inherited behavioral responses are perceived by sensory neurons. The only ciliated cells in *C. elegans* are sensory neurons. Cilia are common organelles in eukaryotic cells. There are two types of cilia, motile cilia and non-motile-cilia, also called primary cilia. Primary cilia are usually found in sensory neurons and responsible for sensory transduction, whereas motile cilia usually cause cell motility (Marshall and Nonaka, 2006). The structure of cilia consists of an axoneme, which is a microtubule-based cytoskeleton. Primary cilia have an axoneme with a ring of 9 outer doublets (9+0 axoneme), whereas the axoneme of motile cilia has two additional inner microtubule singlets (9+2 axoneme). 60 neurons, out of the total number of 302 *C. elegans* has, are ciliated.

1.4.4.3.1.1. Amphid and phasmid neurons

Amphid and phasmid neurons belong to the ciliated neurons. *C. elegans* has 12 amphid sensory neurons, 11 of those are chemosensory neurons, one, AFD, is a thermosensory neuron and ASH shows additional mechanosensory functions (Ward et al., 1975).

The amphids are paired structures at the anterior of the worm and are the main chemosensory organ of *C. elegans*. Amphidial neuron cell bodies are located in the anterior part of the worm in the area of the pharyngeal bulb and connect through axons to the nerve ring. Their dendrites reach to the anterior end of the animal, terminating in ciliated structures in the amphids. The phasmids belong to the primary chemosensory neurons, besides the amphids, and are located in the posterior end of the worm (Hall and Russell, 1991).

Sheath cells protect the end regions of the amphid cilia. The cilia continue into a channel made by socket cells, leaving their endings exposed to the environment, able to detect external stimuli. The amphid neurons differ from other head sensory neurons in the cuticle lining of the socket cells. Most of the amphid neurons show single shaped rods (ASE, ASG, ASH, ASI, ASJ, ASK) or paired rods (ADF, ADL). Some are called wing neurons because of their membrane elaborations and unusual shapes (AWA, AWB, AWD) and the last one is the amphid finger neuron (AFD). This amphid neuron is separate from the other 11 ones in the sheath cell. Its dendritic membrane extends in the sheath cell into 50 villi, named fingers.

The amphid cilium is structured in a distal segment, middle segment, transition zone, neuron/sheath junction and the main dendrite (Figure 1.9)

(Perkins et al., 1986). The shown inner singlets in the *C. elegans* cilia differ from the ones in motile cilia (Chalfie and Thomson, 1982). The phasmid cilia PHA and PHB have a similar structure to the rod-like cilia in amhids (Hall and Russell, 1991).

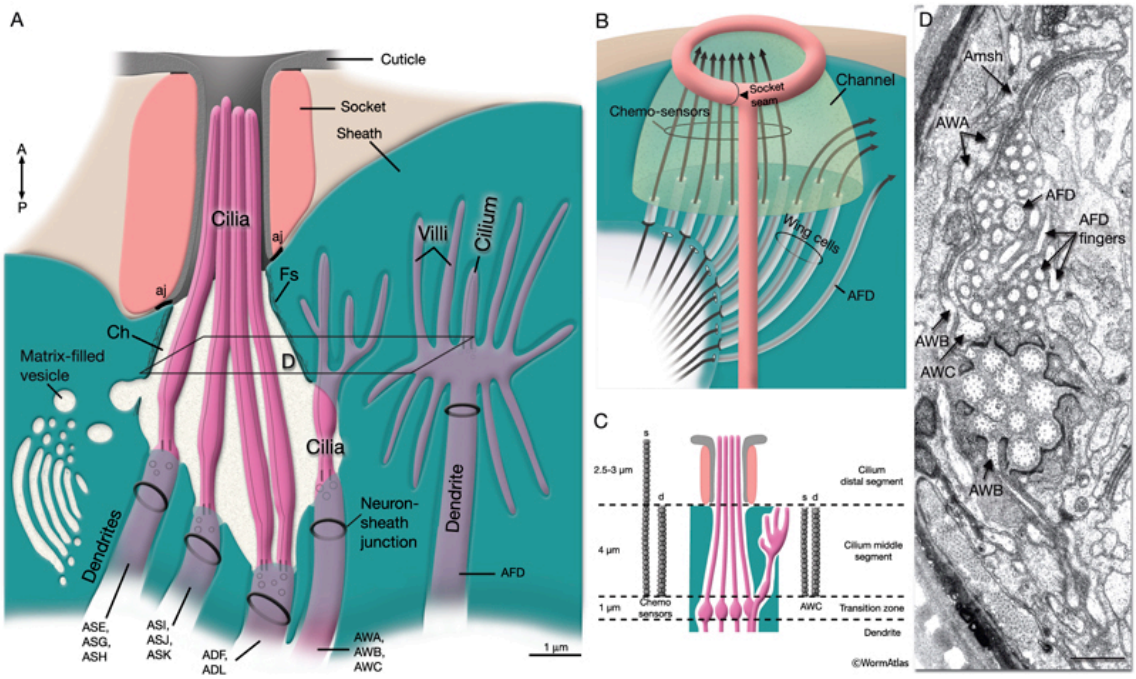


Figure 1.9: Structure of amphids. A. Examples of channel and wing cilia. **B.** Invagination of sheath-cell cytoplasm by amphid dendrites. The AFD neuron remains stable in the sheath cell, whereas all others enter the matrix-filled amphid channel. The cilia of the wing cells penetrate the sheath cell, whereas the eight channel cilia, responsible for chemosensation, are exposed to the outside through the cuticle pore. **C.** Schematic structure of amphid cilia. Each cilium is ~ 7.5 μm long in adult *C. elegans* and consists of three segments: the proximal, middle, and distal segment. **D.** TEM of amphid channel cilia. Transverse section through middle segments of cilia. Bar, 0.5 μm. (Reproduced from Wormatlas.org et al. and Perkins et al., 1986)

1.4.4.4. Dye-filling defective (Dyf) phenotype

To understand the nervous system of *C. elegans* better, especially its ciliary functions, ciliary mutants are used. An easy way to detect sensory cilia mutations is to test their ability to take up dye from their environment. The fluorescent dyes 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI), 3,3'-dioctadecyloxa-carbocyanine perchlorate (DiO) and fluorescein isothiocyanate (FITC) stain the amphid neurons ADF, ASH, ASI, ASJ, ASK, ADL and the two phasmids PHA and PHB (Hedgecock et al., 1985; Herman, 1984).

Laser ablation of or mutations affecting these sensory neurons led to a failure in dye uptake (Perkins et al., 1986). The exact mechanism by which the cilia take up the dye is unclear, but it is assumed to happen via the exposed ciliated endings (Perkins et al., 1986; Starich et al., 1995). Mutants with an inability in taking up dyes are called dye filling (Dyf) defective mutants. However, the Dyf phenotype describes not only worms with abnormal cilia formation. Mutations causing abnormal formation of neuron sheath and socket cells, blocking the cilias access to environment, are included too. Examples of *dyf* mutations in cilia are shown in Figure 1.10.

An example of the Dyf phenotype are *daf-6* mutants (Perens and Shaham, 2005). On the other hand, the lack of a Dyf mutant phenotype does not guarantee a normal cilium structure or function, as the *ifta-2* mutant shows. This mutant is long-lived and defective in dauer formation, showing two ciliary mutant phenotypes (Schafer et al., 2006).

Additional tests detecting abnormal cilia formation are behavioral studies. One example is the osmotic avoidance abnormal (Osm) assay,

revealing the Osm mutant phenotype. Those mutants fail to avoid areas with high, harmful osmotic strengths (Culotti and Russell, 1978). Another phenotype associated with cilia defects is the chemotaxis (Che) phenotype. Here the animals show an inability to react to harmful or non-harmful compounds (Bargmann et al., 1993; Ward, 1973). Studies showed that all of the *osm* mutants and some *che* and *daf* ones fail to take up dye (Perkins et al., 1986). The majority of such sensory mutants are Daf-d at lower temperatures (20-25°C), but exhibit a weak Daf-c phenotype at higher temperatures (Ailion and Thomas, 2000).

1.4.5. *C. elegans* life cycle

C. elegans has a reproductive cycle of around three days under optimal conditions at 20°C. It exists as a hermaphrodite with a 0.05% occurrence of males. The hermaphrodites produce oocytes and sperm and can reproduce by self-fertilization. Males can fertilize hermaphrodites and in this case the male sperm is preferred over the hermaphrodite ones. The brood size of a self-fertilized hermaphrodite can consist of up to 300 eggs. The offspring hatch and develop through four different larval stages (L1-L4), which are separated by molts (see Figure 1.11).

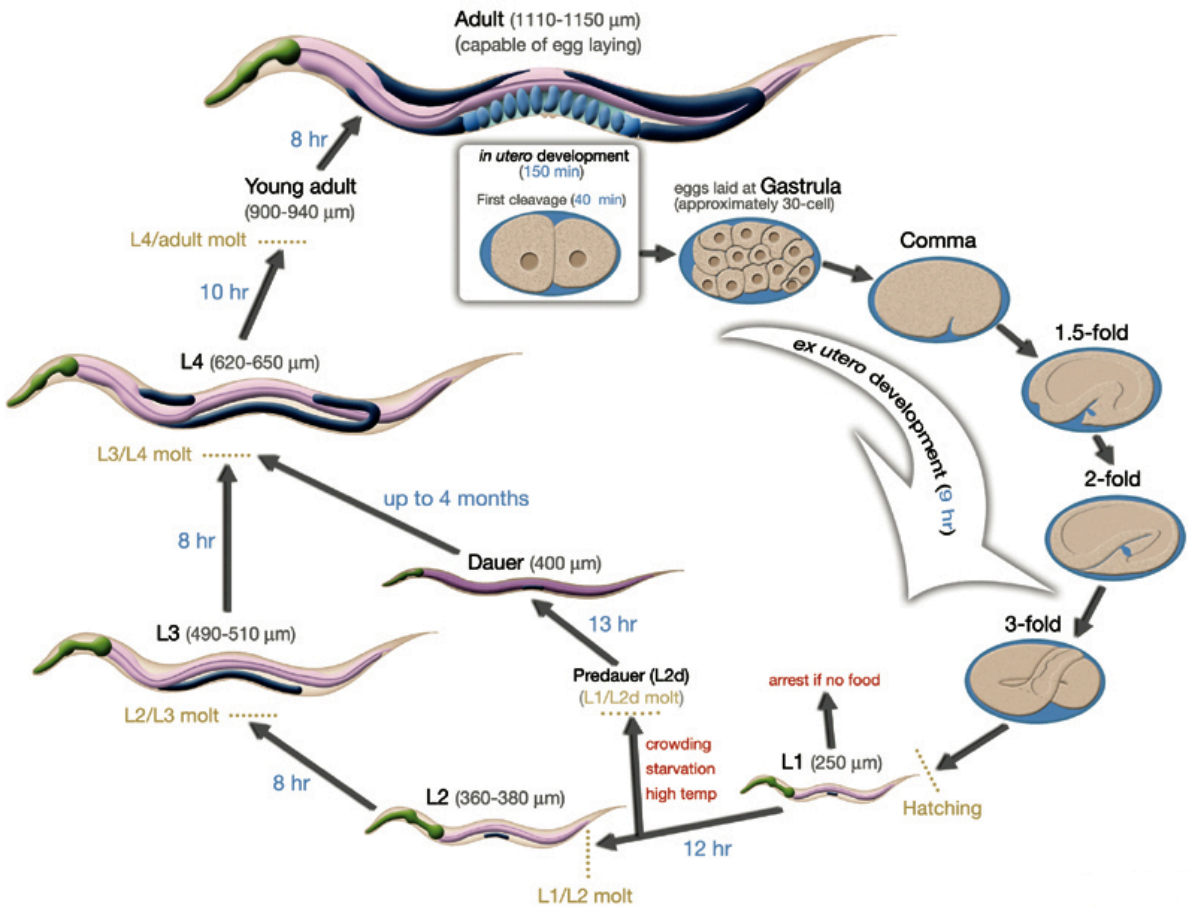


Figure 1.10: Life cycle of *C. elegans* at 22°C. 0 min is fertilization. The numbers in blue next to the arrows indicate the length of time the animal spends at a certain stage (Reproduced from Wormatlas.org et al.).

C. elegans development and life span are temperature-dependent (Byerly et al., 1976). Given standard laboratory conditions at 20°C, wildtype *C. elegans* worms have an average life span of 18-20 days (Gems and Riddle, 1995). Between L1 and L2 stage, *C. elegans* can take an alternative developmental way, the dauer diapause.

1.4.6. Dauer larvae

During development, environmental cues like limited food availability, high population density, high temperatures and/or dauer pheromones, can

cause *C. elegans* to take an alternative developmental pathway at the L2 molt, which results in an arrested stage, the so-called dauer larva diapause (L2d) (see Figure 1.11) (Golden and Riddle, 1984; Golden and Riddle, 1982). The entry and exit from the dauer stage are developmental responses to specific chemosensory cues detected by amphid neurons. These cues inform the larva whether there is sufficient food available to support its reproduction or not. Arrested in this stage, the worms are thinner with a thickened cuticle that makes them look darker (see Figure 1.12). They do not eat, do not move, are more resistant to stress, and can survive for up to four months (Cassada and Russell, 1975).

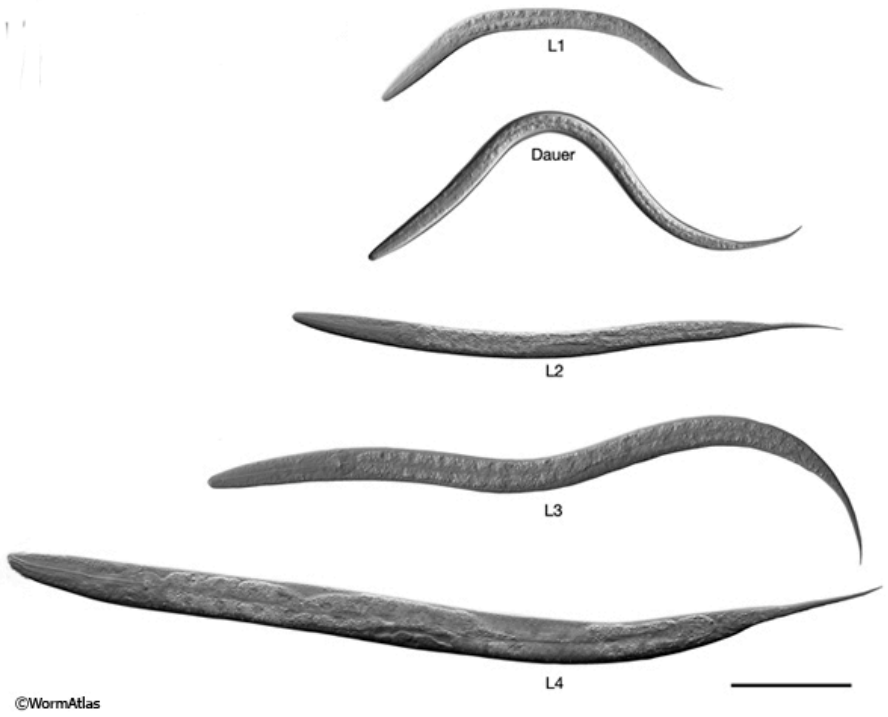


Figure 1.11: Dauer larva compared to the other *C. elegans* larval stages. View with Nomarski microscopy. Scale bar shows 0.1mm (Reproduced from Wormatlas.org et al.).

In the presence of food, the dauer larva continues with the normal development and shows a normal life span afterwards (Riddle, 1988). When worms hatch into environments without any available food source, they arrest in L1 stage diapause (L1d) and are unable to further develop or access into dauer larva (Baugh and Sternberg, 2006).

1.4.6.1. Mutations in abnormal dauer formation (*daf*) genes

More than 30 genes controlling dauer formation have been identified so far. Mutations in *daf* (dauer formation) genes result in the inability to form dauer larvae in response to crowding and starvation (dauer-defective, or Daf-d) or in the formation of dauer larvae under non-dauer-inducing conditions (dauer-constitutive, or Daf-c). Temperature-sensitive dauer-constitutive mutants form dauer larvae only at restrictive temperatures (Albert and Riddle, 1988; Riddle et al., 1981).

1.4.7. The insulin IGF-1 signaling (IIS) pathway in *C. elegans*

In favorable conditions, DAF-2, the homolog of the insulin and IGF-1 receptors, is activated via ligand binding and initiates a downstream signaling cascade that activates AGE-1/aap-1, a phosphatidylinositol 3-kinase (PI(3)K), generates phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Morris et al., 1996). PIP_3 activates the AKT family kinases (Paradis and Ruvkun, 1998) in a 3-phosphoinositide-dependent protein kinase-1 (PDK-1) dependent manner (Paradis et al., 1999). These active AKT family kinases phosphorylate the FOXO class forkhead transcription factor DAF-16 (Henderson and Johnson,

2001; Paradis and Ruvkun, 1998). In doing so, DAF-16 is prevented from entering the nucleus and remains cytoplasmic, suppressing the regulation of many genes required for DAF-2 dependent functions (Lin et al., 1997).

In unfavorable conditions, this IIS pathway is turned off and DAF-16 is not phosphorylated, therefore remains active and present in the nucleus. There it regulates gene expression resulting in increased stress resistance, dauer formation and extended life span. The longevity resulting from mutation of *daf-2* and *age-1* is fully *daf-16* dependent (Dorman et al., 1995; Kenyon et al., 1993). However, retention of DAF-16 in the nucleus is not sufficient to cause the described phenotypes (Lee et al., 2001; Lin et al., 2001). This suggests a much more complicated pathway, potentially involving various other components, such as heat-shock factors and sirtuin protein deacetylases (see section 1.6.) (Berdichevsky et al., 2006; Henderson and Johnson, 2001; Hsu et al., 2003; Wang et al., 2006).

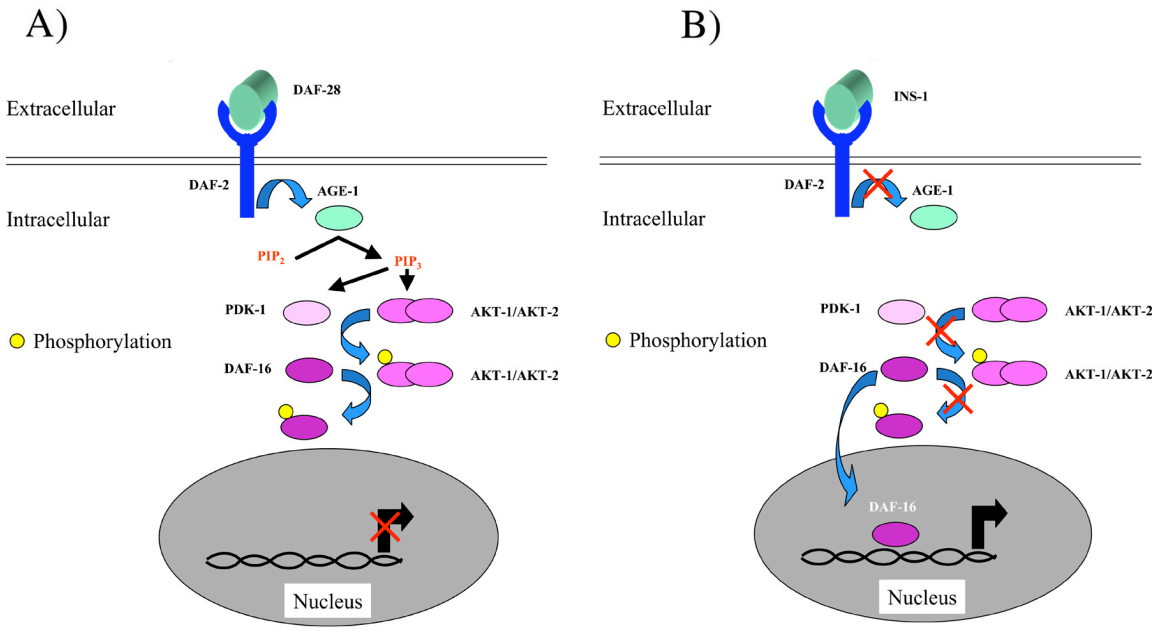


Figure 1.12: IIS pathway in *C. elegans*. A) In favorable conditions, an activating ligand, such as DAF-28, binds to the insulin-like receptor DAF-2 and activates the downstream cascade leding to the phosphorylation of DAF-16 and its retention in the cytoplasm. **B)** In unfavorable conditions, absence of stimulatory ligands, or presence of inhibitory ligands such as INS-1 inhibits the phosphorylation of DAF-16. In this case DAF-16 is active in the nucleus and regulates gene expression of longevity and stress response genes (Reproduced from WormBook (Ewbank, 2006).

How insulin release is regulated in *C. elegans* is not well understood. Assumed key players are environmental cues, since sensory mutants show life span extension, which is *daf-16* dependent (see 1.4.7.) (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999).

Mutations in the insulin/ IGF-1 signaling (IIS) pathway and with that reduced IIS signaling, can cause dauer formation, increased stress resistance and life span extension (Kenyon et al., 1993; Larsen, 1993). Several studies analyzing gene expression in IIS mutants showed that genes responsible for growth and reproduction were down-regulated, whereas genes responsible for stress-defense were up-regulated (Halaschek-Wiener et al., 2005; McElwee et al., 2003; McElwee et al., 2004; Murphy et al., 2003; Oh et al., 2006).

A total of 40 insulin-like ligands have been identified in *C. elegans*, but only a few have been reported to actually affect IIS (Hua et al., 2003; Li et al., 2003; Pierce et al., 2001). Mutations in *daf-2,* the insulin-like receptor, have been shown to extend the life span by up to 150%. Furthermore, *daf-2* mutants still showed high levels of activity in old age, when 90% of the wildtype animals were already immobile, therefore appear to stay more youthful at older ages too (Apfeld and Kenyon, 1998; Kenyon et al., 1993; Kimura et al., 1997). *daf-2* are also resistance to oxidative stress, thermotolerance, resistance to hypoxia, and resistance to bacterial pathogens (Garsin et al., 2003; Riddle and Albert, 1997).

1.4.8. Effect of dye-filling (dyf) defective mutations on *C. elegans* life span

Dyf mutants are typically dauer defective (Starich et al., 1995), yet long-lived and stress resistant. Moreover, longevity is *daf-16* dependent (Apfeld and Kenyon, 1999). Several amphid neurons, such as ADF, ASI, ASG, ASJ, and ASK are involved in dauer entry (Bargmann and Horvitz, 1991; Perkins et al., 1986; Schackwitz et al., 1996; Ward and Miwa, 1978; Ward et al., 1975; Ware, 1975) and ASJ in dauer recovery (Perkins et al., 1986; Ward et al., 1975; Ware, 1975).

Additionally, it is reported that ablation of certain ciliated neurons alone is enough to increase life span in *C. elegans* (Apfeld and Kenyon, 1999) and mutants resistant to oxidative stress are often found to be cilia defective (Fujii et al., 2004). However, not all sensory-impaired *C. elegans* are long-lived.

Some amphid neurons, such as ASI, AWA, and ASG, inhibit longevity, whereas others, such as ASK, promote it (Alcedo and Kenyon, 2004).

1.4.8.1. The Dyf phenotype is not caused by DR

Considering that sensory neurons mediate the movements towards or away from food via chemotaxis, one might think that Dyf mutants are dietary restricted. It is well known that DR extends *C. elegans* life span, exhibiting typical characteristics of DR animals, like abnormal feeding behavior, slower development, thinner body, reduced fecundity (Klass, 1977) or *daf-16* independence (Houthoofd et al., 2003; Lakowski and Hekimi, 1998).

However, this is not the case in Dyf mutants, since sensory mutants do not show any of those characteristics (Apfeld and Kenyon, 1999; Klass, 1977). Therefore it seems unlikely that sensory neurons affect *C. elegans* life span via food consumption (Alcedo and Kenyon, 2004). The more likely scenario is that the IIS pathway is regulated by sensory perception, leding to life span extension in cilia impaired mutants. IIS regulates ageing in C. elegans, but how it does so remains unclear. One possibility is that low IIS leds to reduced oxidative damage. Here a candidate mechanism is up-regulation of antioxidant genes.

1.4.9. Antioxidants in *C. elegans*

Higher eukaryotes have three kinds of SODs, a cytosolic and extracellular CuZn SOD and a mitochondrial Mn SOD, contributing to oxidant defense. *C. elegans* has more isoforms of the these SOD genes. *sod-1, sod-4* and *sod-5*

are isoforms of the cytosolic CuZn SOD (Giglio et al., 1994a; Larsen, 1993), whereas *sod-2* and *sod-3* encode mitochondrial Mn SOD (Giglio et al., 1994b; Hunter et al., 1997; Suzuki et al., 1996).

One study did not detect glutathione peroxidase in *C. elegans*, suggesting that this enzyme is not present in *C. elegans* (Vanfleteren, 1993), as already seen for other invertebrates like *Ascaris* (Lesoon et al., 1990), fleshflies and Chinese oak silk moths (Smith and Shrift, 1979) and house flies (Sohal et al., 1990).

Higher eukaryotes usually have a single catalase isoform. Again, *C. elegans* has three genes encoding catalases, *ctl-1*, *ctl-2* and *ctl-3* (Petriv and Rachubinski, 2004). *clt-1* is a cytosolic catalase, whereas *clt-2* is located in the peroxisomes and is responsible for up to 80% of the total catalase activity in *C. elegans* (Taub et al., 1999). *clt-3* is reported to be localized in the pharyngeal muscle and neurons (Petriv and Rachubinski, 2004).

1.4.9.1. Ferritins in *C. elegans*

Ferritins also contribute to antioxidant defense (see section 1.2.2.5.2.1.). Ferritins in lower organisms are different to those of vertebrates in several respects (section 1.2.2.5.2.). Nematodes, plants, and bacteria have only H-ferritin and also lack the IRE-IRP regulatory system (Arnaud et al., 2007; Gourley et al., 2003; Kim et al., 2004).

C. elegans has two ferritins, FTN-1 and FTN-2, that like mammalian ferritin H subunits contain ferroxidase active sites. Mutations in *ftn-1* cause a decrease in *C. elegans* life span in the presence of excess iron, implying a protective role of *ftn-1* to iron stress (Kim et al., 2004). *ftn-1* expression is

mainly located in the anterior (pharyngeal intestinal valve) and distal part (proctodeum) of the intestine. *ftn-2* expression is located in the pharynx, body wall muscle, hypodermis and the intestine (Cha'on et al., 2007; Kim et al., 2004; McKay et al., 2003; Romney et al., 2008). A recent study showed a role of *ftn-2* in immune defense in *C. elegans* (Simonsen et al., 2011).

1.4.9.2. Ferritin regulation in *C. elegans*

It was guessed that *aco-1* might be the *C. elegans* homologue to IRP-1 based on its aconitase activity. However, the fact that no sequences corresponding to IRPs could be found, and that no binding of ACO-1 to *ftn-1* or *ftn-2* could be detected suggests that, as seen in other invertebrates, no IRP-IRE system exists in *C. elegans* (Gourley et al., 2003). Therefore expression of both ferritins is iron regulated, although *ftn-2* seems to be less responsive to iron levels than *ftn-1* (Romney et al., 2008). Thus the synthesis of both ferritins is induced when iron levels are high to produce enough molecules to accommodate the excess iron. Conversely, the *ftn-1* expression is reduced when iron is low in order to make it available for enzyme synthesis (Gourley et al., 2003).

Not much is known about the regulation of this iron storage protein in the nematode. A recent study examined the promoter regions of both ferritins in *C. elegans* and *C. briggsae* and identified a highly conserved 63 bp sequence as an iron-dependent enhancer of ferritin expression. This sequence was called the “iron-dependent element” (IDE). It contains two GATA motifs and three direct repeats (DR1, DR2, DR3). Deletion of this element caused an inhibition in ferritin expression in low or high iron

conditions, confirming its role as enhancer (Romney et al., 2008). GATA sequences are reported to regulate expression of intestinal-specific genes (Fukushige et al., 1998; McGhee et al., 2007; Moilanen et al., 1999; Oskouian et al., 2005).

Romney et al. found that the *C. elegans* gene *elt-2* binds to the IDE in the 63 bp element and regulates *ftn-1* expression. ELT-2 encodes a GATA-type transcription factor. Their findings suggest that during high iron conditions, ELT-2 reacts with a repeat-binding protein to bind the GATA sequence in the IDE and induce *ftn-1* (and probably *ftn-2*) expression (see Figure 1.15). New results from this lab indicate that iron sensing in *C. elegans* is mediated by the hypoxia inducing factor (HIF) signaling pathway (Ackerman and Gems, 2011).

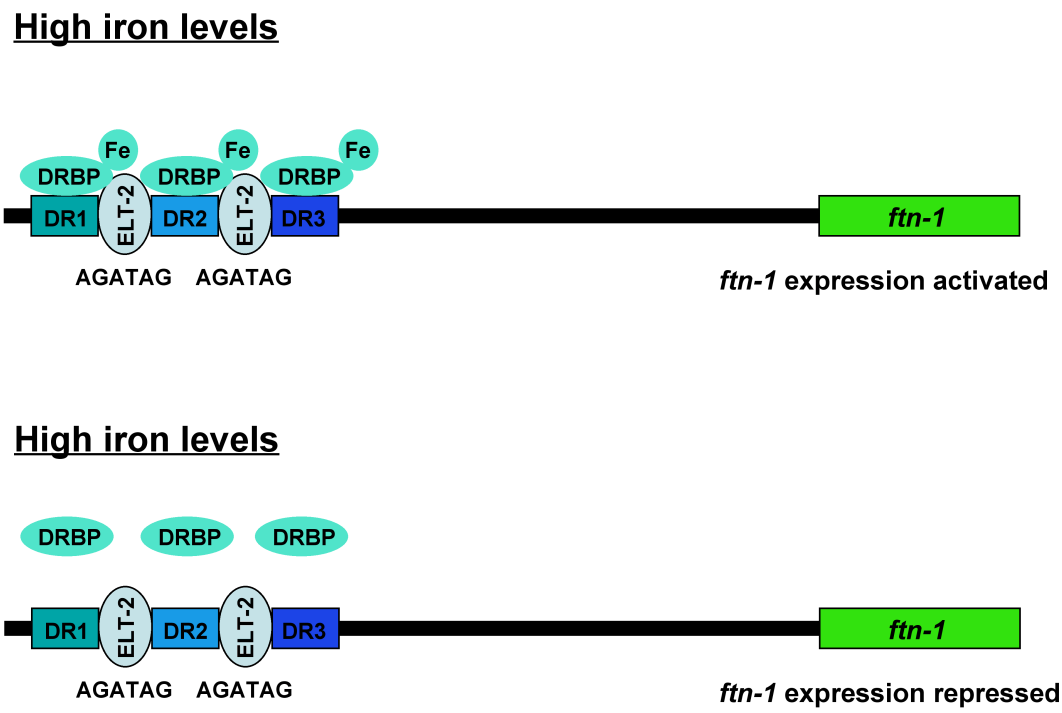


Figure 1.13: Regulation of *ftn-1* in high and low iron conditions. A) Iron causes an interaction between direct-repeat binding proteins (DRBP) and ELT-2, which increases the binding of ELT-2 to the GATA sequence of IDE and activates *ftn-1* expression. **B)** The interaction of DRBP and ELT-2 is reduced during low iron conditions, leding to a repression of *ftn-1* expression (Reproduced from (Romney et al., 2008).

1.4.9.3. The role of ferritins in oxidative damage in *C. elegans*

If oxidative damage causes ageing, and iron generates oxidative stress, proteins such as ferritin that contribute to iron homeostasis might also protect against ageing. Loss of *ftn-1* reduces life span still further in iron stressed worms (Kim et al., 2004) and excess iron shortens their life span (Gourley et al., 2003). An effect of loss of *ftn-1* in ageing in non-stressed worms has not been reported yet. Therefore *ftn-1* is important for iron homeostasis affecting life span during iron stress conditions in *C. elegans*. However, iron homeostasis proteins might protect against ageing since *ftn-1* and *mtl-1*, are strongly up-regulated in long-lived *daf-2* mutants. So far, it is not known whether enhanced iron homeostasis or reduced free iron levels

protects against the ageing process. I investigated this connection during my PhD and the results are shown in Chapter 3 and 4.

1.4.10. Agents used to induce oxidative stress in *C. elegans*

A widely used approach to detect changes in overall levels of antioxidant defense is to measure resistance to oxidative stress. Here, I list some of more commonly used agents to induce oxidative stress in *C. elegans*, and some findings pertaining to them.

1.4.10.1. Hyperoxia

60% oxygen decreases *C. elegans* mean life span by 17% (Honda et al., 1993; Honda and Matsuo, 1992). This can readily be achieved by maintaining *C. elegans* on petri dishes in an oxygen chamber (Honda et al., 1993; Honda et al., 2008).

1.4.10.2. Redox cycling agents

Paraquat, or *N,N'*-dimethyl-4,4'-bipyridinium dichloride, is a commonly used herbicide. Experimentally it is used to induce oxidative stress thanks to its ability to produce intracellular superoxide. A concentration of 2 mM paraquat is just sufficient to increase cyanide-independent oxygen consumption (a measure of $O2^{\bullet -}$ generation) and also modestly decreases *C. elegans* life span (Keaney et al., 2004).

Juglone (5-hydroxy-1,4-naphthalenedione), is a natural product extracted from walnut trees. Like paraquat, this natural naphthoquinone can

reduce oxygen, thus creating superoxide radicals. Juglone is also used as an oxidative stress inducer in *C. elegans* (Bolton et al., 2000; Van Raamsdonk and Hekimi, 2009; Vanfleteren, 1993).

1.4.10.3. Peroxides

Peroxides, such as hydrogen peroxide and *tert*-butylhydroperoxide, can react with transition metals and generate ROS via the Fenton reaction.

$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH\bullet + OH^-$$

$$Fe^{2+} + (CH_3)_3\,COOH \Rightarrow Fe^{3+} + (CH_3)_3\,CO\bullet + OH^-$$

For part of my thesis work, relating to iron homeostasis and its role in oxidative damage and ageing, I used *tert*-butylhydroperoxide (TBH) to test stress resistance in my worms (An and Blackwell, 2003) (see Chapter 3 and 4).

1.5. Arguments against the oxidative damage theory

A positive correlation between ageing and oxidative damage has been seen in many different species, supporting this theory as a major cause of ageing (Muller et al., 2007). However, a more powerful way to interrogate this theory is by increasing or decreasing oxidative damage levels in different organisms and test the resulting effect on ageing. According the theory, increased oxidative damage should led to accelerated ageing, while reduced oxidative damage should slow down ageing. To manipulate ROS levels, manipulation of enzymatic antioxidant levels are often used. However, this

approach has generated controversy in that some studies show the predicted effect of manipulating antioxidant levels, whereas others do not.

For instance, some studies have shown that over-expression of CuZn SOD and Mn SOD causes life span extensions in *Drosophila* and catalase over-expression has been reported to have the same effect in mice (Parkes et al., 1998; Schriner et al., 2005; Sun et al., 2002; Sun and Tower, 1999). By contrast, other studies saw no effect on life span in mice and *Drosophila* upon SOD over-expression, but increased fly resistance to oxidative stress (Huang et al., 2000; Mockett et al., 1999; Orr and Sohal, 1992; Orr and Sohal, 1993; Reveillaud et al., 1991; Seto et al., 1990). However, there are several other findings that argue against the oxidative damage theory.

For instance, reduced ROS production in flies does not increase their life span (Miwa et al., 2004), but shortens it (Bayne et al., 2005). The longest-living rodent species, the naked mole-rat (*Heterocephalus glaber*), shows elevated oxidative damage compared to mice (Perez et al., 2009). Mice fed with antioxidants showed reduced oxidative damage without having any effect on ageing (Comfort et al., 1971; Holloszy, 1998). Another drawback for the oxidative damage theory is the finding that SOD2 mutant mice have elevated oxidative damage levels without showing any effect on ageing (Van Remmen et al., 2003). Moreover, antioxidant supplementation in humans, such as E and beta-carotene, has been reported to have life span decreasing effects (Bjelakovic et al., 2007; Bjelakovic et al., 2004).

1.5.1. Antioxidant defense and ageing in *C. elegans*

Loss of the Mn SOD, *sod-2* increased oxidative damage and the sensitivity to oxidative stress induced via paraquat, whereas loss *sod-3* had no effect. Individual mutations in one of both had no effect on resistance to hyperoxia, but loss of both resulted in hypersensitivity to hyperoxia (Doonan et al., 2008; Honda et al., 2010; Van Raamsdonk and Hekimi, 2009). Despite this, loss of function mutations in either or both Mn SOD genes had no effect on life span (Chavez et al., 2007; Doonan et al., 2008; Honda et al., 2008; Van Raamsdonk and Hekimi, 2009; Yang et al., 2007; Yen et al., 2009). One study even saw an increase in life span after *sod-2* deletion (Van Raamsdonk and Hekimi, 2009).

Loss of function in the CuZn SOD, *sod-1* increased sensitivity to paraquat, but that of *sod-5* did not. None of the CuZn SODs seem to play a role in the response reaction to hyperoxia. Knock down of *sod-1* caused a slightly shortened life span in three studies (Honda et al., 2008; Yang et al., 2007; Yen et al., 2009) but had no effect in two other studies (Van Raamsdonk and Hekimi, 2009; Yen et al., 2009). Deletion of *sod-4* (extracellular) and *sod-5* showed no effect on life span (Doonan et al., 2008; Honda et al., 2008; Van Raamsdonk and Hekimi, 2009; Yen et al., 2009).

Loss of function in the *C. elegans* gene *ctl-1* that encodes a cytosolic catalase did not show any effect on life span, whereas loss of function of *ctl-2* shortened it (Olahova et al., 2008; Petriv and Rachubinski, 2004). Simultaneous over-expression of all three catalase genes did not increase *C. elegans* life span (Doonan et al., 2008).

The effect of antioxidant supplementation on ageing in *C. elegans* are variable. Some studies saw life span extension after antioxidant supplementation (Benedetti et al., 2008; Harrington and Harley, 1988; Ishii et al., 2004), whereas others did not (Adachi and Ishii, 2000; Schulz et al., 2007). It remains unclear whether any of the effects seen are attributable to reduced oxidative damage.

1.5.2. Antioxidants and ageing in long-lived IIS mutants

In long-lived *daf-2* and *age-1* mutants SOD activity is higher than wildtype (at older ages), suggesting a possible role of antioxidant defense in longevity assurance (Honda and Honda, 1999). Both mutant types have higher levels of SOD and catalase and are resistant to oxidative stress (Honda and Honda, 1999; Honda et al., 2010; Larsen, 1993; Vanfleteren, 1993). These findings are consistent with the oxidative damage theory of ageing.

Loss of one of the Mn SODs *sod-2* or *sod-3* has no effect on the resistance to oxidative stress in these IIS mutants, but loss of both reduces resistance (Honda et al., 2010). However, deletion loss of either or both genes has no effect on life span in *daf-2* mutants (Doonan et al., 2008; Honda et al., 2010; Van Raamsdonk and Hekimi, 2009). By contrast, a small decrease in *daf-2* mutant life span was seen after *sod-3* RNAi (Honda et al., 2008; Murphy et al., 2003). Possibly this reflects off-target RNAi effects. One study saw a life span increase in *daf-2* mutants after deletion of *sod-3* (Honda et al., 2008). Turning to the role of the cytosolic SOD in *daf-2* mutants, loss of *sod-1* slightly shortened their life span, whereas *sod-5* had no effect (Doonan et al., 2008;

Yang et al., 2007). Loss of the extracellular SOD, *sod-4*, increased *daf-2* mutant life span, probably by reducing IIS (Doonan et al., 2008; Honda et al., 2008).

Overall, the research testing the oxidative damage theory of ageing by manipulating enzymatic antioxidants or antioxidant supplementation in *C. elegans* has thus far failed to provide convincing support for this prominent theory. Here I further test the theory by investigating the influence on ageing of iron homeostasis, levels of free iron and, by inference, the Fenton reaction. I used iron chelators and ferritin levels to alter free iron levels and examine the effects on ageing (see Chapter 3 and 4).

1.6. Sirtuins and their role in ageing

My second project during my PhD related to the role of situins in *C. elegans* ageing. Sirtuins belong to the silent mating type information regulator family (SIR). Several studies have indicated an involvement of sirtuins in life span regulation (Howitz et al., 2003; Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001), and also insulin secretion (Moynihan et al., 2005), stress response (Anderson et al., 2003; Brunet et al., 2004; Cohen et al., 2004), axonal degeneration (Araki et al., 2004), transcription factor activity (Muth et al., 2001), rDNA recombination (Gottlieb and Esposito, 1989; McMurray and Gottschling, 2003; Smith and Boeke, 1997), regulating enzyme activity (Starai et al., 2002) and apoptosis (Motta et al., 2004; Vaziri et al., 2001).

The early *SIR* mutant experiments played an important role in establishing the idea of transcriptional silencing (Sauve et al., 2006). Experiments in *Salmonella typhimurium* showed that cobB, a bacterial sirtuin, is both a deacetylase and a ADP-ribosyl transferase (Tsang and Escalante-Semerena, 1998). Meanwhile, there has been extensive work involving sirtuins and several studies show that the deacetylation reaction is conserved throughout the phyla from archea and eubacteria to yeast, mouse and humans (Imai et al., 2000; Landry et al., 2000; Min et al., 2001; Sauve et al., 2001; Smith et al., 2000; Tanner et al., 2000), as is their ADP-ribosyl transferase activity (Frye, 1999).

1.6.1. Histone deacetylases (HDAC)

To keep the massive amount of genetic information stored in the DNA more compact, DNA is wrapped around proteins, including core histones H2A, H2B, H3 and H4 and structured into nucleosomes. These line up like a “thread on a spindle” and form the units of chromatin, which pack the large eukaryotic genome as chromosomes into the nucleus (Figure 1.15).

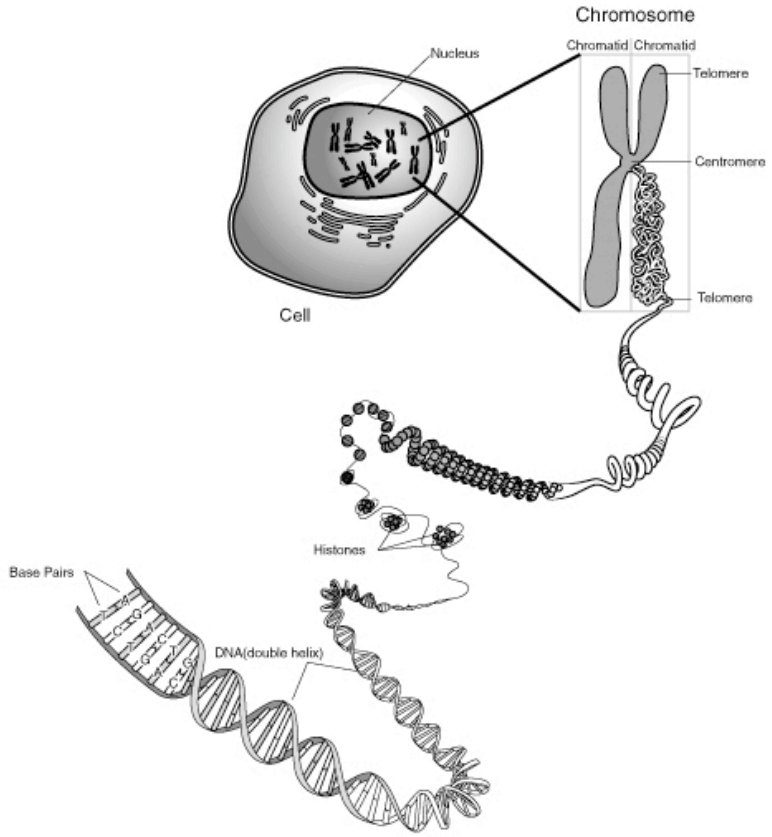


Figure 1.14: From DNA to chromosome. This figure shows the chromosomes within the nucleus of a cell, as well as its formation. The base pairs of two single strands of DNA connect via hydrogen bonds to double strand DNA and an alpha helix is formed. The DNA winds around proteins, the histones, and builds so called nucleosomes. Those line up to build the chromatins, whose ends are called the Telomeres and whose middle piece, the centromere, connects it to a second chromatin to form the chromosome (Image reproduced via NCBI from the *National Human Genome Research Institute* (NHGRI)).

Eukaryotic cells pack their genetic information into large linear chromosomes in the nucleus, whereas prokaryotic cells show smaller circular chromosomes in the cytoplasm. Chromatin can be found in a condensed (heterochromatin) or extended (euchromatin) form. Through chromatin status, gene expression and DNA replication can be controlled (Griffiths et al., 2000). A switch between the two different chromatin forms can be effected by changes in methylation or acetylation of histones. HDACs and histone acetyltransferases (HATs) are the main enzymes controlling histone methylation and acetylation status. Protein acetylation/deacetylation is an important post-translational modification that rivals phosphorylation/dephosphorylation in the regulation of protein activity (Kouzarides, 2000; Yang, 2007).

1.6.1.1. Classes of histone deacetylases (HDAC)

HDACs fall according to function and DNA homology into two families and four different classes. The families are the classical HDACs and the *s*ilent information regulator-2 (Sir2)-related proteins (sirtuin) families. In humans, class I, II and IV belong to the classical HDACs. Class I HDACs (HDAC 1, 2, 3 and 8) are homologs of the yeast gene “reduced potassium dependency 3” (Rpd3) and are mainly located in the nucleus, but also the cytoplasm (HDAC 3). Class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) are homologs to the yeast “histone deacetylases 1” (hda1) and are located in the nucleus and cytoplasm. Class II HDACs are twice as large as the class I HDACs and both are inhibited by Trichostatin A. HDAC 11 belongs to class IV (Gregoretti et al., 2004). Class I, II and IV HDACs require Zn^{2+} for deacetylase activity (Yang,

2007). Class III HDACs include SIRT1-7. These are nicotine adenine dinucleotide $(NAD)^+$-dependent enzymes, located in nucleus and cytoplasm and are homologs of the yeast “silent information regulator 2” (*sir2*) gene. This class of HDACs has no sequence resemblance to the classical family, but like classical HDACs they show ADP-ribosyl transferase activity (Tanny et al., 1999).

1.6.1.2. Chromatin silencing mechanism

Through amine groups on lysine and arginine residues, histones are positively charged. The positive charges allow the histones to interact with the negatively charged phosphate groups on the DNA backbone. HATs acetylate the ε-amino groups of lysine and neutralize the positive charges on the histone by changing amines into amides. This decreases the ability of the histones to bind to DNA, resulting in chromatin expansion, which allows transcription and therefore gene expression (Braunstein et al., 1996). Thus increased acetylation is associated with the euchromatin form. HDACs on the other hand remove acetyl groups from ε-amino groups located near the amino termini of histones. This leads to an increased positive charge on the histone tails. A strong binding between the histones and DNA backbone condenses the DNA structure and makes this region of chromatin structure inaccessible, suppressing gene expression (Figure 1.16) (Bi and Broach, 1997; Braunstein et al., 1993; Loo and Rine, 1994). Therefore deacetylation promotes heterochromatin form. HDACs are very conserved enzymes, found in both eukaryotes and prokaryotes (Frye, 2000).

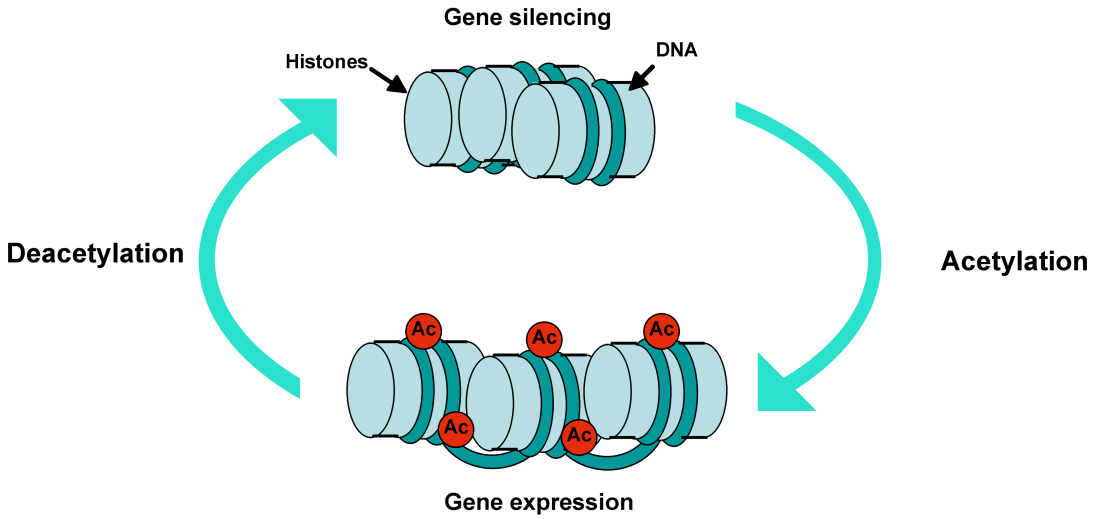


Figure 1.15: Acetylation and deacetylation of histones (Image reproduced from Riken Research (Yoshida, 2008). HATs acetylate histones, which leds to chromatin expansion and gene expression. On the other hand, deacetylation by HDACs removes the acetyl groups that led to increase binding of the DNA to the histones. This high affinity binding results in chromatin condensation and gene silencing.

1.6.1.3. Catalytic mechanism of sirtuins

The catalytic mechanism of sirtuins is different to those of the other HDACs. For instance, unlike all other HDACs, sirtuins are NAD^+-dependent. The NAD^+-dependency of *SIR2* became evident from the fact that for each deacetylated lysine one NAD^+ molecule is cleaved (Landry et al., 2000; Tanny and Moazed, 2001). The reaction catalyzed by sirtuins removes an acetyl moiety from the ε-amino group of lysine residues in protein targets (Imai et al., 2000). During this process nicotinamide and O-acetyl-ADP-ribose (AADPR) are generated. AADPR is the product of the transfer of an acetyl group from the substrate to the ADP-ribose (ADPR) of NAD^+ (Tanny and Moazed, 2001) (Figure 1.17). NADH is not only a product of the *SIR2* deacetylation reaction, but also an inhibitor of sirtuins (Bitterman et al., 2002; Landry et al., 2000;

Sauve et al., 2005; Sauve and Schramm, 2003), whereas NAD^+ is a sirtuin activator.

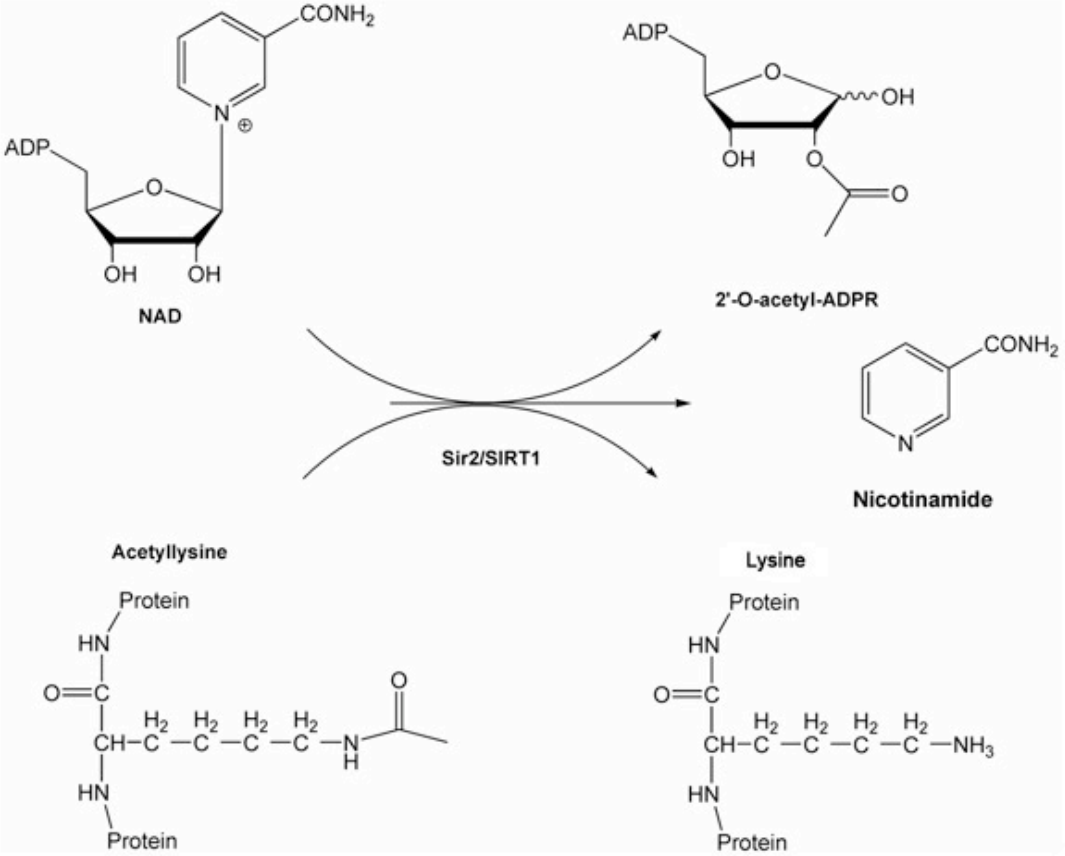


Figure 1.16: Stoichiometry of the NAD^+ dependent Sir2/SIRT1 reaction. An acetylated protein reacts with NAD^+ and an acetyl group transfer from the substrate to the ADP-ribose (ADPR) generates O-acetyl-ADP-ribose (AADPR) and nicotinamide (Reproduced from (Yang and Sauve, 2006).

The requirement for the high energy-cofactor NAD^+ not only sets the sirtuins apart from the other HDACs, but also links them to the ribosyl transferases (Landry et al., 2000; Min et al., 2001; Sauve and Schramm, 2003; Sauve et al., 2006). The ADP-ribosyl transferases (ARTs) modify proteins posttranslationally by adding one or more ADPR moieties (Belenky et al., 2007; Ziegler, 2000). These enzymes are involved in various cell

processes like DNA repair and apoptosis (Berger et al., 2004; Corda and Di Girolamo, 2003). One reason for the NAD^+ involvement in the deacetylation reaction of sirtuins might be the synthesis of AADPR. AADPR is a second messenger that can link deacetylation to cellular metabolism (Sauve et al., 2006) and maybe silencing (Tanner et al., 2000). Furthermore, the NAD^+-dependency of sirtuins provided an initial conceptual link to DR effects (see section 1.6.3.).

1.6.2. The role of sirtuins in ageing in different model organisms

The role of sirtuins in ageing has been investigated in several model organisms. For a period, it was presented as something of a miracle gene, causing life span increases in various species and therefore showing the potential to do so in humans too (Guarente, 2003). Here, I review the sirtuin-ageing story, including various controversies that have arisen.

1.6.2.1. How sirtuins became a major topic in biogerontology

It all started in the budding yeast *Saccharomyces cerevisiae*. In 1979 the genes *SIR1*, *SIR2*, *SIR3* and *SIR4* were first identified in connection to silencing at the silent mating type loci, HML and HMR in yeast (Rine et al., 1979) and telomeres (Ivy et al., 1985). Interestingly, loss of silencing by the *SIR* complex at the mating type loci caused sterility in old yeast cells, resulting in a novel phenotypical marker for yeast ageing (Smeal et al., 1996). Moreover, mutations in the *SIR* complex shortened replicative yeast life span

(Kaeberlein et al., 1999; Kennedy et al., 1995). This could imply gene silencing promotes longevity.

The accumulation of extra-chromosomal DNA circles (ERCs) was proposed to be a major contributor to replicative ageing in yeast (Sinclair and Guarente, 1997). ERCs are self-replicating episomes, which accumulate in old mother cells. One possibility is that accumulation of ERCs cause ageing through diluting components of the replication or transcriptional machinery. ERCs are generated via homologous recombination in rDNA tandem arrays of genes, which are asymmetrically retained in the mother cell during the cell cycle. *SIR2* was reported to inhibit recombination in the rDNA (Gottlieb and Esposito, 1989; Rine and Herskowitz, 1987). It was published in 1999 that over-expression of *SIR2* increases yeast life span, strengthening the view that *SIR2* is an important determinant in yeast ageing (Kaeberlein et al., 1999).

1.6.2.2. Effects of sirtuins on yeast ageing are context specific

SIR2 over-expression does not always increase yeast life span. *SIR2* over-expression in a different background strain than previously used, did not increase yeast life span (Kaeberlein et al., 2004), although *SIR2* over-expression always caused an increase in silencing at the rDNA genes in all strains tested (Fritze et al., 1997; Smith et al., 2007; Smith et al., 1998).

In terms of chronological life span (CLS), deletion of *SIR2* in two long-lived mutants increased it, whereas *SIR2* over-expression decreased it (Fabrizio et al., 2005). However, the significance of this to ageing is unclear, since it was recently discovered that CLS is limited by a drop in medium pH caused largely by acetic acid secretion (Kaeberlein, 2010).

1.6.2.3. The role of sirtuins in *Drosophila* life span

In 2004 it was reported that over-expression of *dSir2* in Drosophila results in a life span increase compared to the controls used. *dSir2* over-expressing lines, showing 4-fold increase in *dSir2* mRNA levels, had a reported life span extension of up to 57% (Bauer et al., 2009; Rogina and Helfand, 2004). Homozygous mutations in *dSir2* are reported to shorten fly life span (Astrom et al., 2003) as did reduced *dSir2* expression in the nervous system induced by RNAi (Kusama et al., 2006). On the other hand, transheterozygote and heterozygote flies for *dSir2* mutations show no effect on life span (Newman et al., 2002; Pallos et al., 2008).

However, recent findings challenge the role of *dSir2* in *Drosophila* longevity. According to new studies, the life span increase is abrogated after out-crossing and inclusion of the appropriate genetic controls of the reported *dSir2* over-expressing lines. Further generated *dSir2* over-expresser lines, with higher *dSir2* levels than the original lines, also failed to show an increase in life span. These results imply that *dSir2* over-expression alone does not increase *Drosophila* life span (Burnett et al., 2011).

1.6.2.4. The role of sirtuins in *C. elegans* ageing

The involvement of *SIR2* in yeast replicative life span leds to the question of whether effects of sirtuins of ageing are evolutionary conserved. After all, ERCs do not accumulate in worms, flies or mammals, thus are not determinants of ageing in higher organisms (Marciniak et al., 1998). *C. elegans* has four homologues to the yeast *SIR2* and the one with the highest level of identity is called *sir-2.1*.

1.6.2.4.1. *sir-2.1* acts downstream of *daf-16* in longevity assurance

In 2001 it was reported that over-expression of *sir-2.1* increases *C. elegans* life span in a manner dependent on *daf-16*, the FOXO transcription factor regulated by the insulin/ IGF-1 (IIS) pathway (Tissenbaum and Guarente, 2001). In 2005 it was published that, although connected to the IIS pathway through DAF-16, *sir-2.1* is not required for the regulation of life span by this pathway, suggesting that *sir-2.1* acts in a parallel pathway to IIS, converging at the point of DAF-16 regulation (Viswanathan et al., 2005). Another study identified two binding partners of *sir-2.1* in the family of 14-3-3 proteins (Berdichevsky et al., 2006). These proteins belong to a conserved family of regulatory molecules. The mammalian 14-3-3 proteins interact with the FOXO transcription factor, the mammalian homologue of *C. elegans* DAF-16 (Brunet et al., 1999), causing retention of FOXO in the cytoplasm (Fu et al., 2000; Tzivion et al., 2001; Van Der Heide et al., 2004). The increased life span and resistance to heat and oxidative stress induced by *sir-2.1* over-expression was dependent on the 14-3-3 proteins *par-5* and *ftt-2* but they had no effect on *daf-2* mutants. Therefore, the following mechanism was suggested: the 14-3-3 proteins bind to phosphorylated *daf-16*, retain it in the cytoplasm and upon stress initiate the binding of DAF-16 and SIR-2.1 and relocation to the nucleus to activate stress resistance and extension of life span (Wang et al., 2006). Furthermore, *sir-2.1(ok434)* mutants show decreased life span and reduced resistance towards stresses, such as hydrogen peroxide, heat shock and UV irradiation (Wang and Tissenbaum, 2006). All these findings support the role of *sir-2.1* as an anti-ageing gene in *C. elegans*. However, mounting controversy in the sirtuin ageing field (see

below) led me to further investigate the role of *sir-2.1* in *C. elegans* ageing (see Chapter 5 and 6).

1.6.3. Do sirtuins mediate the effects of dietary restriction (DR) on life span?

DR increases life span and delays age-related diseases in different model organism, and consequently is a highly investigated subject in biogerontology. Since not many people could tolerate DR in exchange for the beneficial effects, there is an interest in developing drugs to mimic the effects of DR. To achieve that, the mechanism of DR needs to be better understood. Since all sirtuins are NAD^+ dependent and NAD^+ is important in metabolism, a link to DR was proposed (Imai et al., 2000; Smith et al., 2000). The reduced form of NAD^+, NADH, is also an effective sirtuin inhibitor. Therefore the increased NAD^+: NADH ratio during DR suggested the view that sirtuins are up-regulated and therefore involved in the beneficial DR effects (see below).

1.6.3.1. Sirtuins and their involvement in DR effects in yeast

In 2000 sirtuins were first reported to be mediators of DR in yeast (Lin et al., 2000). Based on the catalytic mechanism of histone deacetylases, two hypotheses were developed to explain the involvement of sirtuins in the DR induced life span extension in yeast.

Supporting the rate of living theory of ageing, the first hypothesis suggested the metabolic change from fermentation to respiration that happens during DR as an explanation. An increase in *SIR2* activity by

elevated NAD^+ levels was proposed to induce a metabolic shift (Imai et al., 2000; Lin et al., 2002; Smith et al., 2000; Su-ju Lin, 2004). The second hypothesis challenged the first one by indicating that NAD^+ levels were not changed by DR and proposed that DR effects in yeast are mainly caused by decreased NADH concentrations caused by up-regulation of the Pnc1 enzyme (Anderson et al., 2003). Consistent with both ideas, *SIR2* mutants were reported to show no life span extension upon DR (Lin et al., 2000).

1.6.3.2. Findings questioning the role of sirtuins in DR effects in yeast

Theories involving the NAD^+: NADH ratio are challenged by the finding that DR can extend life span in respiratory-deficient yeast (Kaeberlein et al., 2005a; Kaeberlein and Powers, 2007). Several studies independently reported that DR does not effect rDNA recombination in a *SIR2* dependent way (Smith et al., 2009a) and DR does not increase *SIR2* activity (Kaeberlein et al., 2005a; Kaeberlein and Powers, 2007; Kaeberlein et al., 2005c; Riesen and Morgan, 2009; Smith et al., 2009a) nor is the increase in life span by DR inhibited by the deletion of *SIR2* or all the sirtuin proteins (Fabrizio et al., 2005; Jiang et al., 2000; Kaeberlein et al., 2004; Kaeberlein et al., 2006a; Kaeberlein et al., 2006b; Lamming et al., 2005; Smith et al., 2007; Tsuchiya et al., 2006). Furthermore, *SIR2* over-expression and DR showed an additive effect in yeast life span, suggesting that the two mechanisms work through different pathways. Another proof against *SIR2* as a DR mediator is the finding that a *SIR2* deletion strain showed even further life span increase than its controls (Kaeberlein et al., 2006b). Considering all the recently published data, it seems unlikely that *SIR2* plays a significant role in the DR dependent

increase in life span in yeast. Kennedy and Kaeberlein suggested a new model for life span extension upon DR: the TOR signaling pathway. Here reduced TOR expression leds to the expected DR effects in a sirtuin independent fashion and this increases both replicative and chronological life span in yeast (Kaeberlein et al., 2005c).

1.6.3.3. The role of sirtuins in the DR response in *Drosophila*

DR is also able to increase life span of this model organism, including the typical DR characteristics like reduced weight and fecundity and increased activity and resistance to oxidative stress and damage (Bross et al., 2005; Parashar and Rogina, 2009; Zheng et al., 2005). It was reported that the life span extension upon DR in *Drosophila* is *dSir2* dependent. Here, flies lacking *dSir2* did not show a life span increase upon DR. Furthermore, *dSir2* mRNA levels were elevated in flies subjected to DR and there was no additive effect on life span in DR treated *dSir2* over-expressing flies (Parashar and Rogina, 2009; Rogina and Helfand, 2004; Rogina et al., 2002).

However, newer studies showed that flies homozygous for a nullimorphic *dSir2* deletion allele responded to DR in the same way as isogenic wildtype. This shows that the DR effect in *Drosophila* is *dSir2* independent (Burnett et al., 2011).

1.6.3.4. The role of sirtuins in the DR response in *C. elegans*

These findings led to the question of whether the involvement of sirtuins in DR mediated life span extension are conserved in other species or restricted to yeast only.

A single study suggested an involvement of sirtuins in the life span extension during DR in *C. elegans* (Wang and Tissenbaum, 2006). In this study two alleles of the *eat-2* mutation, *eat-2(ad465)* and *eat-2(ad1113)*, and *unc-13*, *unc-13(s69)* and *unc-13(e450)*, were crossed into the *sir-2.1(ok434)* background. The *eat-2* (EATing defective) and *unc-13* (UNCoodinated movement) mutations exhibit an abnormal pharyngeal pumping phenotype due to defects in the pharynx and can be used as a means to exert DR (Kohn et al., 2000; Lakowski and Hekimi, 1998). Their findings suggested that the increased life span in *eat-2* and *unc-13* mutants was *sir-2.1* dependent (Wang and Tissenbaum, 2006).

However, several other studies found that mutation of *sir-2.1* mutants does not suppress the effect of DR on life span (Greer and Brunet, 2009; Hansen et al., 2007; Honjoh et al., 2009; Lee et al., 2006; Schulz et al., 2007).

1.6.3.5. The role of sirtuins in mammalian DR responses

SIRT1 has been linked to energy metabolism and the response to DR in mice. SIRT1 knockout mice showed a smaller body size and reduced activity than their wildtype counterparts while consuming more food and oxygen. Furthermore, they did not show the same beneficial effects of DR, such as increased activity and life span, as wildtype animals. Instead of maintaining metabolic rate during DR, SIRT1 null mice showed a reduction in

their metabolic rate. Thus, mice lacking SIRT1 seem to be unable to adjust to DR conditions (Boily et al., 2008). This inability was not caused by differences in perception of food restriction: the typical physiological changes upon DR, reduction of glucose levels, insulin-like growth factor 1 (IGF1) and triglycerides, occured as expected.

These DR associated phenotypes were also seen in transgenic mice over-expressing SIRT1. This data could imply that SIRT1 is involved in the beneficial effect of DR in mammals. However, although SIRT1 over-expressing mice seem to show typical characteristics of DR, one essential phenotype is missing: the increase in life span (Bordone et al., 2007; Chen et al., 2005). So far no data shows that SIRT1 over-expression can increase mammalian life span.

1.6.4. The resveratrol story: sirtuin activator or not?

After the presentation of evidence that sirtuins in the DR response, the possibility that sirtuin activators could act as DR mimetics was pursued. An in vitro screen in 2003 identified resveratrol as a sirtuin activator, able to increase SIRT1 activity 13-fold and *SIR2* activity 2-fold (Howitz et al., 2003). Thus resveratrol seemed a potential DR mimetic. Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic phytoallexin produced in various plants after infection with bacteria or fungi. One of the best-known sources of resveratrol is in grape berry skins and it is also present at very low levels in red wine.

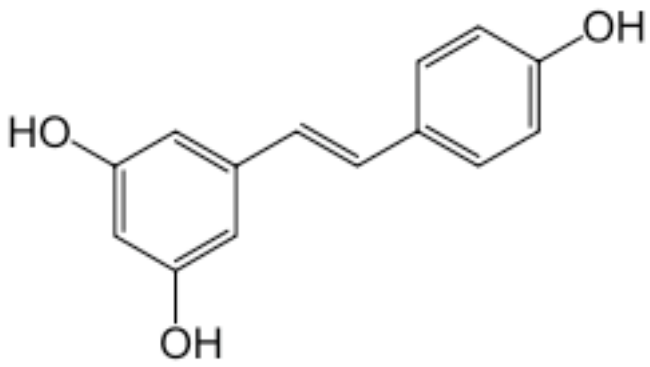


Figure 1.17: The chemical structure of the polyphenol resveratrol ($C_{14}H_{12}O_3$)
(Reproduced from MDidea)

Since its discovery in 1939, resveratrol has been connected to many beneficial outcomes such as cancer prevention in mice (Baur and Sinclair, 2006; Jang et al., 1997) and in human cells (Baur and Sinclair, 2006), neuroprotective effects (Anekonda, 2006; Karuppagounder et al., 2009; Kumar et al., 2006; Sharma and Gupta, 2002), anti-inflammatory effects (Elmali et al., 2007; Gentilli et al., 2001; Tsai et al., 1999), cardioprotective effects (Kopp, 1998; Szmitko and Verma, 2005), anti-diabetic effects (Deng et al., 2008; Palsamy and Subramanian, 2008; Su et al., 2006), anti-oxidative DNA damage effects (Mizutani et al., 2001) and anti-viral effects (Docherty et al., 2006; Heredia et al., 2000; Palamara et al., 2005). Furthermore, it is reported that resveratrol can increase the survival of mice on a high fat diet (Baur et al., 2006) and exposure to low concentrations of resveratrol resulted in an increased cell survival after DNA damage in human cells (Howitz et al., 2003).

Then, it was reported that resveratrol can increase life span in the yeast *Saccharomyces cerevisiae* (Howitz et al., 2003), the nematode *Caenorhabditis elegans* (Viswanathan et al., 2005) and the fruit fly *Drosophila*

melanogaster (Bauer et al., 2004; Wood et al., 2004). Thus far the only positive effect of resveratrol on life span in vertebrates was found in 2006 in the short-lived fish *Nothobranchius furzeri* (Valenzano et al., 2006). Life span extension is not the only reported DR feature that resveratrol induces: resveratrol induces transcriptional changes similar to those induced by DR (Barger et al., 2008; Pearson et al., 2008; Smith et al., 2009b). Furthermore, treatment with resveratrol of HdhQ111mammalian neuronal cells, a model for Huntington's disease, protected cells against cytotoxicity in a SIRT1 dependent way (Parker et al., 2005).

1.6.4.1. Findings arguing against resveratrol as a sirtuin activator

Newer studies question the role of resveratrol as sirtuin activator. To determine the activity of sirtuins in sirtuin activator screens, a Fluor de Lys assay was used. Here, the deacetylation rate of a chemically modified acetylated peptide, the Fluor de Lys-SIRT1 peptide, is measured. During deacetylation, the peptide is proteolytically cleaved which causes fluorescence (Wegener et al., 2003). Fluorescence increased 8-fold in the presence of resveratrol, implying that resveratrol is a sirtuin activator (Howitz et al., 2003). However, other studies found that if a different sirtuin activity assay was used (^{3}H-acetate release assay), resveratrol did not activate sirtuin activity. Moreover, it transpired that the activation effect in the Fluor de Lys assay was dependent on the fluorophore (Borra et al., 2005; Kaeberlein et al., 2005b). This clearly implies that resveratrol is not a sirtuin activator. Consistent with this, resveratrol failed to increase *SIR2* activity in three different yeast strains (Kaeberlein et al., 2005b) and does neither increased

rDNA silencing nor decreased rDNA recombination (both indicators of *SIR2* activity in yeast) (Howitz et al., 2003; Kaeberlein et al., 2005b).

1.6.4.2. Resveratrol does not increase life span in a sirtuin dependent manner

Recent studies showed that the effect of resveratrol on life span are not as robust as first presented (Bass et al., 2007; Beher et al., 2009; Borra et al., 2005; Kaeberlein et al., 2005a; Kaeberlein et al., 2005b). In the case of *Drosophila*, the life span increase after resveratrol treatment was not reproducible and the effect of resveratrol in yeast was strain-specific (Bass et al., 2007; Kaeberlein et al., 2005b), whereas a slight effect of resveratrol on life span in *C. elegans* could be reproduced in some trials, but not in others. Notably, even *sir-2.1* mutant worms showed a slight increase in life span in some trials, which is consistent with the view that resveratrol is not an activator of sirtuins (at least not directly) (Bass et al., 2007).

Furthermore, it seems that the reported life span extensions via resveratrol in *C. elegans* is controlled through a different pathway than the one caused by elevated *sir-2.1*. This is shown by the *daf-16* independence of the resveratrol-induced life span extension and the fact that resveratrol causes life span extensions by up-regulating genes that are down-regulated by *sir-2.1* over-expression. This result implies an inhibiting effect of resveratrol on sirtuin activity instead of the so far assumed activating effect (Viswanathan et al., 2005).

In mice, resveratrol treatment results in decreased levels of inflammation, proteinuria and cataracts and increased median life span in

mice on high fat diet (Baur et al., 2006). However, there are no reported effects on life span of resveratrol treatments on mice with a normal diet (Pearson et al., 2008). Furthermore, resveratrol failed to induce other DR phenotypes, such as reduced heart rate or decreased core body temperature (Mayers et al., 2009).

1.6.5. The hyping of sirtuins to the public

Based on the view that sirtuins protect against ageing, the pharmaceutical industry took an interest in them. Cosmetics companies such as Estee Lauder, Clinique, Avon and REN all currently market resveratrol-containing products, ostensibly as an anti-ageing agent. In 2004 the company Sirtris Pharmaceuticals was founded to exploit the commercial potential of sirtuins and went public in 2007. To avoid the hardship of maintaining a DR lifestyle, the company is developing orally available drugs that activate sirtuins, thereby mimicking DR in order to treat diseases where ageing is a risk factor, including type 2 diabetes. Sirtuin activators include resveratrol formulations, e.g. SRT501, plus other sirtuin activators such as SRT1720, SRT2183, SRT1460, SRT2104 and SRT2379.

The research Sirtris is conducting is based particularly on the work of D. Sinclair of Harvard Medical School, a co-founder of Sirtris. The Guarente lab published in 1999 that extra copies of *SIR2* increase yeast life span (Kaeberlein et al., 1999). In 2003, Sinclair reported that resveratrol activates sirtuins and, by doing so can increase life span in yeast (Howitz et al., 2003), *C. elegans* and *Drosophila* (Wood et al., 2004) (see section 1.6.4.). In 2007 Sinclair was involved in a new screen for SIRT1 activators and found three

compounds, SRT1720, SRT2183 and SRT1460 to be structurally different from and 1000-fold more potent than resveratrol. A mouse study found that resveratrol treatment results in gene expression pattern resembling those observed during DR (Smith et al., 2009b). Here, treatment of mice kept on a high fat diet with the synthetic resveratrol compounds SRT1720 and SRT501 reduced the blood glucose levels by enhancing the insulin caused glucose disposal. However, the resveratrol treatment had no effect on life span in mice with normal diet (Miller et al., 2011; Milne et al., 2007).

Despite a series of publications questioning the very basis of the Sirtris research: that resveratrol is an activator of sirtuins (Beher et al., 2009; Borra et al., 2005; Garber, 2008; Kaeberlein and Kennedy, 2007; Kaeberlein et al., 2005b; Pacholec et al., 2010)(see section 1.6.4.1.), in 2008 the massive Pharma Company GlaxoSmithKline (GSK) bought Sirtris Pharmaceuticals for $720 million.

Given that two different studies showed that the SIRT1 activation through resveratrol is dependent on the fluorophore used in the activity assay, a new study tested the Sirtris series. Again, Sirtris compounds proved not to activate the native SIRT1 using assays other than the Fluor de Lys. Furthermore the previously reported reduced blood-glucose level of obese mice given resveratrol was not reproducible (Pacholec et al., 2010), contrary to a Sirtris-linked report (Milne et al., 2007). Here, mice fed with the Sirtris compound SRT1720 ate more and gained weight compared to control animals (Beher et al., 2009; Kaeberlein and Kennedy, 2007; Pacholec et al.; Pacholec et al., 2010). GSK also recently had to stop a phase two trial of

SRT501, a formulation of resveratrol, in myeloma patients because some showed kidney failure (Staff, 2010).

After all the published reports arguing for and against role of sirtuins in ageing, Richard Miller from the University of Michigan commented: “If I were asked to list ten proteins that deserve a lot of attention in ageing in mammals, the sirtuins would be on that list. They just wouldn’t be at the top of the list” (Buchen, 2010; Patricia F. Dimond, 2010). A goal of my work was to verify the previously reported role of *sir-2.1* in *C. elegans* ageing (see Chapter 5 and 6).

Chapter 2: Material & Methods

2.1. Materials

2.1.1. Reagents

LB (Luria-Bertani) Medium

Dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl

in H_2O, and adjust to 1 L, adjust pH to 7.5 with NaOH and autoclave.

OP50 growth solution

Dissolve 5 g trytone, 2.5 g yeast extract in H_2O,

and adjust to 1 L and autoclave.

M9 solution

Dissolve 7 g $Na_2HPO_4.2H_2O$, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4.7H_2O$, in

H_2O, add H_2O to 1 L and autoclave.

Freezing medium

Dissolve 20 ml 1M NaCl, 10 ml 1M KH_2PO_4 (pH 6.0), 60 ml 100% glycerol in

H_2O, add H_2O to 1 L, autoclave and add 0.6 ml 0.1 M $MgSO_4$.

Phosphate Buffered Saline (PBS)

Dilute 10 x PBS (100 ml + 900 ml H_2O) and add 1 ml Tween-20.

Running Buffer (10x)

Dissolve 30.3 g tris-base, 144 g glycine and 10 g SDS in H_2O and adjust volume to 1L. For 1x concentration take 100 ml 10 x Running Buffer and add 900 ml H_2O.

Western Blocking Solution

1 x PBS + 0.1% Tween-20 + 5% milk powder or 1% bovine serum albumin (BSA). Prepare 200 ml: add 200 µl Tween-20 and 10 g of milk powder to 200ml PBS or 2 g BSA.

Western Transfer Buffer (10 x)

Dissolve 144 g glycine and 30.2 g tris-base in 800 ml sterile H_2O and adjust volume to 1L. For 1 x concentration use 200 ml of 10 x buffer (or 200 ml of 5 x buffer), 200 ml ethanol (100%) and add 1600 ml H_2O. Transfer Buffer can be used up to 3 x in a row, but keep refrigerated!

Protein Buffer

150 mM KCl, 1mM EDTA, 0.25% SDS, 1.0% NP-40, 50 mM Tris/HCl pH 7.4, and Roche Complete protease inhibitors). Alternatively, use Sigma CelLytic for mammalian cells + Protease inhibitors (this works just as well).

LB (Luria-Bertani) agar

Dissolve 10 g Bacto-tryptone, 5 g Bacto yeast, 5 g NaCl, 15 g agar in H_2O and adjust volume to 1L.

Nematode growth media (NGM)

Dissolve 3 g NaCl, 20 g agar and 2.5 g peptone in H_2O

and adjust volume to 1 L and autoclave. Then add 1 ml 1 M Ca Cl_2, 1 ml 1 M $MgSO_4$, 1 ml 5 mg/ml cholesterol (in ethanol) and 25 ml 1M KH_2PO_4.

NGM Isopropyl-ß-D-1-thiogalactopyranoside (IPTG) agar

Dissolve 3 g NaCl, 20 g agar and 2.5 g peptone in H_2O and adjust volume to 1 L and autoclave. Then add 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 1 ml 5 mg/ml cholesterol, 25 ml 1M KH_2PO_4, 400 µl 50 mg/ml carbenicillin and 1 ml 1M IPTG.

2.1.2. *C. elegans* strains used

N2, obtained from the Caenorhabditis Genetic Center (CGC), was used as wildtype throughout this thesis. All other strains used in the experiments presented in this thesis are described and listed in the corresponding chapter. Strains used as control to mutant animals, carrying the same genetic background, but have the wildtype version of the gene mutated in the mutant strain, are also referred to as wildtype.

2.2. Methods

2.2.1. Microscopy

All *C. elegans* life span, stress assay and maintenance observations were performed using a Leica dissecting microscope (model MZ95). For detecting transgenic animals carrying a GFP marker, the Nikon fluorescent dissecting microscope (model SMZ1000) attached to the Nikon super high-pressure mercury lamp power supply was used.

2.2.2. Maintenance of stocks

Unless stated otherwise, worms were grown at 20°C in 60 mm Petri culture dishes on nematode growth media (NGM), with OP50 *Escherichia coli* bacteria as food source. The growth of OP50 is limited on NGM agar through its uracil auxotrophy. To avoid starvation and provide a permanent food source the worms were transferred several times a week. Here two methods were used: a fast and convenient way of transferring worms is “chunking”. This technique uses a sterilized scalpel to cut out a chunk of agar containing worms and transfers it to a fresh seeded plate. This method was often used to gain a big mixed staged colony (L1-adult worms). Another method is to pick single animals with the help of a thin platinum wire (worm pick) to a fresh plate. This method is a more controlled way to get a certain amount and stage of offspring, since the development and fertility of *C. elegans* is well known. In both cases a bunsen burner was used to sterilize the worm pick/scalpel before use. The frequency of the transfer depended

also on the temperature the worms were kept and the genotype. Wildtype stocks, while in use, were subcultured every 3-4 days. Brood size measurements were performed by transferring several individual worms during their reproductive period and count the eggs produced.

2.2.2.1. Maintenance of extrachromosomal transgenic lines

In the case of no-integrated transgenic lines, such as GA904, GA901 (see Chapter 4) and GA707 (see Chapter 5), the worms needed to be maintained by picking 3-4 single worms with the corresponding transgenic phenotype to fresh plates several times a week. Because of instability of the transgene array during cell division not all animals inherit the transgene array from transgene parents. Without selecting every few generations, the proportion of transgenic animals would become more and more diluted by animals not carrying the transgene, which might end in losing the transgenic line.

2.2.3. Decontamination and synchronization of nematode stocks

Sometimes, *C. elegans* stocks acquire bacteria, yeast or fungal contaminants. To get rid of low-level fungal contaminants, one can chunk or transfer single worms from a “clean” area of the contaminated plate to a fresh plate. Bacterial contamination on the other hand requires a more aggressive treatment with alkaline hypochlorite, called egg prep.

2.2.3.1. Alkaline hypochlorite treatment (egg prep)

This method kills all contaminants, and worms that are not protected by the eggshell. To gain a high yield of eggs, several plates crowded with gravid adult worms were washed off the agar plates with M9 buffer and transferred in a volume of 200 µl to an eppendorf microcentrifuge tube using a glass pipette. The glass pipette helped reducing the loss of biomass caused by worms stuck to the walls of plastic tips. 150 µl bleach solution (7:8 sodium hypochlorite: 4M NaOH) was added and incubated for 4-5 minutes. Filling the tube with M9 buffer stopped the reaction. A centrifugation for 1 min at 2500 rpm followed. The supernatant was removed and the tube re-filled with M9 buffer. The pellet was dissociated completely with vigorous shaking. This washing step was repeated twice more before the egg pellet was transferred to freshly seeded plate. This method was also used to gain synchronized colonies, since all the worms hatching from the bleached eggs have the same age.

2.2.3.2. Egg lay

Another method to get synchronized *C. elegans* cultures is an egg lay. Here, several gravid worms are transferred for a certain amount of time on fresh plates to let them lay eggs. The time and number of worms depended on the desired outcome. For instance, 20-30 gravid worms were placed over night on 100 mm NGM plates for Western/oxyblot samples. To gain enough biomass for EPR samples (see below), several plates of each strain were prepared in that way.

2.2.4. Life span measurements

Generally, life spans are influenced by interaction of environmental factors with the genetic background of the individual. Usually 125-150 age synchronous hermaphrodite worms (25-30 worms per plate) were transferred to the corresponding plates (five NGM, DF, FAC or RNAi plates) and counted every second day. Worms without *glp-4(bn2)* of *rrf-3(pk1426)* mutation (these worms are sterile at 25°C) were transferred to fresh plates every day during the first week, to avoid mixing the older worms of the life span trial with their offspring. In case of 5-fluoro-2'-deoxyuridine (FUdR) usage, one day before the start of the life span experiment FUdR was applied topically to the surface of the plates in a final concentration of 10 µM. FUdR blocks DNA replication, thereby preventing progeny production. The L4 stage was used as t=0 for life span analysis. When a worm failed to move after touching, it was removed from the plate and scored as dead. Animals that crawled off the plate, burrowed, stuck to the wall and/or lid, showed uterine rapture or bagged (i.e. died from internal hatching of embryos) were censored.

2.2.5. GFP measurement

To quantify the GFP expression in the *Pftn-1::GFP* reporter strain (see Chapter 3 and 4), 40 young adult worms were picked in a 96 well V-shaped microtitre plate (Greiner) (Wolff et al., 2006). GFP was measured in the Genios plus plate reader (Tecan) at the excitation and emission wavelengths of 395 nm and 535 nm, respectively.

2.2.6. Stress assays

2.2.6.1. Iron treatments

All iron treatments were performed on NGM plates with added ammonium iron (III) citrate (Sigma), also called ammonium ferric citrate (FAC) ($C_6H_8O_7$ Fe NH_3). Ferric iron (iron (III)) is the stable oxidative state of iron in aerobic conditions, and the normal oxidative state used by cells. In a solution at physiologic pH, iron (III) that is not bound by a chelator or carrier molecule will form iron (III) hydroxide complexes that are virtually insoluble. But the iron (III) in the FAC plates is reduced to ferrous (II) salt by exposure to light. Iron (II) that is free to participate in Fenton chemistry is a major source of oxidative stress. I added the appropriate amount of FAC to the NGM agar shortly before the plates were poured and seeded them with the usual food source, *E. coli* OP50.

2.2.6.2. Oxidative stress assays

Generally, oxidative stress assays include exposure to oxidizing agents for a certain period of time. In *C. elegans* usually oxygen, hydrogen peroxide, paraquat or *tert*-butyl hydroperoxide (TBH) are used to increase oxidative stress in the worms (Blum and Fridovich, 1983). Here, *tert*-butyl hydroperoxide (TBH) was used. For the TBH assays 150 ml NGM were prepared in advance (see NGM protocol). At the day of the assay, the agar was melted and the salts added (see NGM protocol) and TBH in a final concentration of 10 mM TBH was added. After pouring and drying of the plates in the flow hood, the animals were transferred as late L4s/young adults

to the plates and scored for survival every 1-2 hours. Within the first 1-2 h after transfer to the TBH plates, the worms tried to crawl off the plates. Therefore the worms needed to be placed constantly back to the middle of the plates. Afterwards the animals stop moving and can be scored at 1-2 h intervals.

2.2.6.3. High temperature induction of constitutive dauer formation (Hid) assay

The Hid phenotype describes increased dauer formation at temperatures above 25˚C, usually 27˚C (Malone et al., 1996). One cause of Hid is reduced IIS and increased DAF-16 activity. *daf-16* mutants (cannot form dauers, negative control), N2 (makes a small number of dauers at 27ºC) and *age-1(hx546)* (makes many dauers at 27ºC, positive control) were used as controls. The Hid test is very sensitive to the exact temperature in the incubator used. Temperature differences of 0.5ºC or less showed large effects on dauer formation at temperatures near 27ºC. A short egg lay (1-3h) was performed at 20ºC and the eggs were incubated at 20ºC (controls) and 27ºC. Dauer and non dauer formation were counted after 44-55h.

2.2.7. Statistics used

Statistical comparisons of mean life span values were performed using the JMP 7.0.1 programme (2007 SAS Institute Inc.) using the log rank test. For analysis of the GFP-expression measurements the Student's T-test (two-tailed) was used.

2.2.8. Dye-filling defect (Dyf) test

The lid of an Eppendorf microcentrifuge tube was filled with 199 µl M9 buffer and 1 µl of the 1,1'-dioctadecyl-3,3,3', 3'-tetramethylindo-carbocyanine perchlorate dye (DiI) stock solution (2mg/ml) was added. Several worms at different stages were transferred into the lid filled with dye and incubated for 2-3h at room temperature. Afterwards the worms were transferred, using a glass pipette to minimize the loss of worms, to fresh plates and to let them destain by crawling on the bacterial lawn for 1 h. Afterwards they were transferred again to a fresh plates by picking the individual worms. In that way the possibility that the worms crawled in the remaining dye on the plate, reducing a proper destaining could be avoided. This destain was over night and the next day a fluorescence scope was used to checked for the Dyf phenotype.

2.2.9. RNAi by ingestion of dsRNA

2.2.9.1. Mechanism

RNA interference (RNAi) introduces homologous double stranded RNA (dsRNA) and specifically targets a gene's product, resulting in its degradation. This causes suppression of gene expression by preventing translation or transcription of specific genes. The RNA-induced silencing complex (RISC) is the controlling factor in this mechanism and short dsRNA molecules are the starting points. The dsRNA activates the ribonuclease protein dicer (Bernstein et al., 2001) and cleaves dsRNAs to produce double-stranded fragments of

20–25 bp (Zamore et al., 2000). These fragments are called small interfering RNAs (siRNAs). They are separated into single strands, integrated into the active RISC complex and target mRNA to start mRNA cleavage. This prevents the mRNA from being used as a translation template.

2.2.9.2. RNAi feeding protocol

RNAi was performed by feeding the worms HT115 bacteria containing the L4440 vector into which the gene of interest was cloned. The L4440 vector is a modified version of Bluescript and contains a T7 promoter on each side. The HT115 *E. coli* strain contains IPTG-inducible T7 polymerase, which produces RNA transcripts from both T7 promoters, producing dsRNA copies of the gene cloned into the multiple cloning sites. Bacteria carrying only the L4440 plasmid, without any gene of interest cloned into it, were used as a negative control. The RNAi feeding clones were kindly provided from the RNAi library from Weiqing Li (University of Washington), containing clones from the Ahringer and Vidal Library (Kamath et al., 2003). To test for the right insert in the plasmid, the vectors were purified from a 5 ml over night culture using QIAgen Mini-prep according to manufacturer's instructions and sent for sequencing to the Wolfson Institute for Biomedical Research.

An egg prep or egg lay of the strains was applied to the IPTG plates seeded with the different RNAi feeding clones. The resulting animals were denoted as the F_1 generation. An egg lay was performed on the plates with the required RNAi feeding clones to get the F_2 generation. As soon as the synchronised worms reached the L4 stage, the life span measurements were started as described.

2.2.10. Generation of transgenic worms via microinjection

To obtain transgenic worms containing multiple copies of *ftn-1,* the microinjection technique was used. A DNA solution containing the PCR product of the gene of interest together with a genetic marker for selection of transgenic animals was co-injected into the distal gonad syncytium. For all injections the wildtype strain N2 in the young adult stage was used. After several months of unsuccessful tries by myself, David Weinkove obtained several transgenic *ftn-1* over-expresser lines for me, but I myself generated a stable control line, bearing only the *coel::GFP* marker.

2.2.10.1. Amplification of the *ftn-1* fragment

The complete *ftn-1* coding region including 3860 bp upstream and 1048 bp downstream was amplified using *C. elegans* genomic DNA as template. The 3860 bp upstream of the *ftn-1* gene included the promoter region and the downstream part included the 3'UTR. The Phusion High-Fidelity DNA Polymerase PCR system from Finnzymes was used to amplify the 5990 bp long DNA fragment with the following primers:

Forward primer: ftn-1.5in	TGTAGGGTTTGATTGTGGTTTG
Reverse primer: ftn-1.4in_rev2	AAATTCGGAAATGTCGCAGC

The best amplification temperature was 62.8°C.

Table 2.1: PCR components.

Component	Volume (μl)
5x Phusion HF buffer	4
10mM dNTPs	0.4
Phusion DNA polymerase	0.2
Forward primer (100pmol/μl)	1
Reverse primer (100pmol/μl)	1
DNA	1
H_2O	17.4

The amplified *ftn-1* fragment size was 5990bp long.

2.2.10.2. Preparation of the Marker

The *coel::GFP* plasmid (1 ng/μl) was co-injected as scoreable marker with the *ftn-1* PCR product. It is a GFP-marker, which expresses GFP in six cells called the coelomocytes (see Chapter 4). To amplify the marker, the plasmid was transformed into XLI competent cells in the following way: the XLI competent cells were thawed on ice and 20 μl of the marker were added or 20 μl of water (control). These samples were incubated on ice for 10-30 min. Afterwards the transformed cells were seeded to pre-heated (37°C) LB ampicillin plates and incubated at 37°C over night. The next day a transformant colony was picked, and used to inoculate 12 ml LB ampicillin solution. This was incubated at 37°C in an orbital shaker over night. Then this plasmid was purified using the QIAprep spin Miniprep Kit. The method was performed according to manufacturer's directions and the plasmid was sent for sequencing to the Wolfson Institute for Biomedical Research.

2.2.10.3. Further preparations for injection

2μl of the purified marker (266ng/μl) were mixed with 1 μl of the *ftn-1* PCR product (~7.2ng/μl) and 7μl of distilled H_2O was added, giving a final DNA concentration of 59.2ng/μl. Needles were pulled using borosilicate glass capillaries (1.0mm OD, 0.75mm ID) with a P97 micropipette puller (Sutter Instrument Company). Prior to the injection process, the tip was broken by gently tapping the needle a glass slide or by moving the needle against debris on the agarose pad under the microscope. A 2% agarose solution was prepared and one drop was transferred onto a coverslip and a second coverslip was quickly placed on top of it. A few seconds later the agarose was solidified and the two coverslips could be peeled apart. The coverslip pads were dried in a 37°C incubator over night.

The injections were performed on an inverted microscope; model DMIRB (Leica) with a 40x Nomarski non-oil immersion objective equipped with a micromanipulator (Leica) and a Picospritzer II pressurized injection system (General Valve Corporation) with a needle holder. To inject the DNA into the worms pressurized nitrogen gas was used.

2.2.10.4. The injection process

The needle was filled with 1 μl of DNA mix and placed into the needle holder. A drop of oil was placed on an injection pad and one worm was transferred onto it. For injection, usually young adult worms with few or no eggs in the uterus were used. The worm was brought in position and the arm of the syncytial gonad was focused with the 40x objective. Using the

manipulator, the needle tip was moved into the same focal plane bordering to the targeted location. The worm was pushed against the needle tip until the cuticle stretched inwards and the needle inserted into the core.

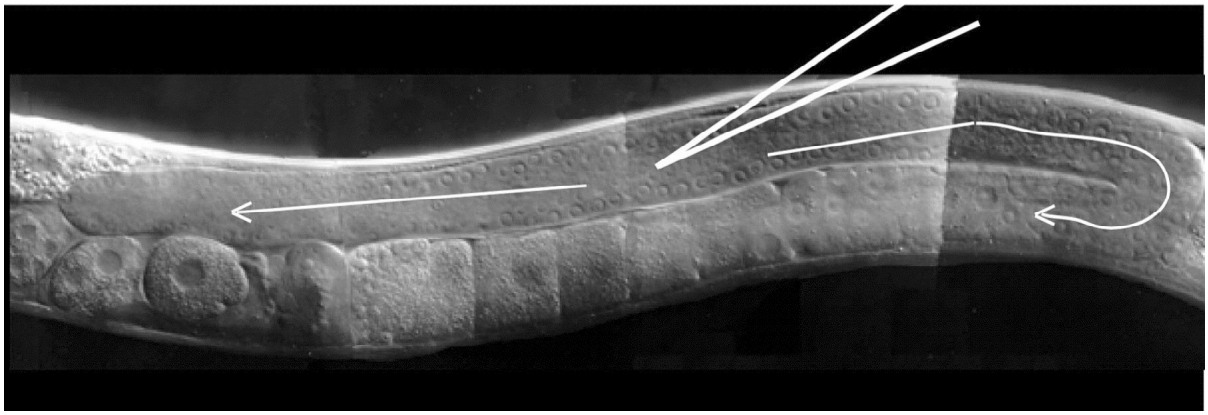


Figure 2.1: Microinjection of the *C. elegans* gonad. The optimal position of the injection needle in the cytoplasmic core of the distal germ line is depicted. For DNA transformation, injection solution should flow in both directions through both the distal and proximal germ line (Reproduced from WormBook (Evans, 2006).

The DNA mixture was injected into the distal arm of the gonad. The pressure of the injection made the solution flow in both directions throughout the gonad until the gonad noticeably swelled. After the injections, one drop of M9 buffer was transferred onto the injected worms, which caused the worms to detach from the agarose pad, recovering in the solution. As soon as the worm swam briskly, it was transferred to a seeded NGM plate. The F_1 generation was then screened for transgenic animals. Even when the injection was successful, not all F_1 transgenics are generally capable of transmitting the transgene, so single transgenic F_1 animals carrying the GFP marker were picked to NGM plates and left to lay eggs at 20°C. As a control line young N2 hermaphrodites were injected with the marker but not with the *ftn-1* DNA fragment.

2.2.11. Classical genetic methods

2.2.11.1. *C. elegans* conventions

C. elegans has six chromosomes, consisting of five autosomes (I-V) and an X chromosome. While hermaphrodites are diploid for all chromosomes, males are diploid for the autosomes, but haploid for the X chromosome. To better understand the crossing schemes presented in this thesis, especially for the mapping in Chapter 5, some standard *C. elegans* conventions are explained here.

Hermaphrodite

Male

$\frac{m}{+}$

This usually describes a heterozygous strain. A mutant version of gene *m* is on one chromosome while a wildtype version of this gene *m* (+) is on the homologous chromosome.

$\frac{dpy\ \ m}{unc}$

dpy and *m* mutation are linked on one chromosome, while *unc* is on the homologous chromosome. *dpy* and *m* are in *cis* position, *unc* is in trans position to *dpy* and *m*. Wildtype copies of *unc* are present on the chromosome of *dpy* and *m* and *dpy* and *m* wildtype copies are present on the chromosome with the *unc* mutation.

$\frac{dpy}{+}\ \frac{unc}{+}$ Both mutations are on different chromosomes and in heterozygous state.

$\frac{m}{m} = m$ When a homozygous mutation is described, it is not necessary to show both chromosomes.

$\frac{m}{+} = \frac{m}{+} \times \frac{m}{+}$

etc. etc.

Since worms can self-fertilize, usually a single arrow is drawn beneath the genotype.

2.2.11.2. Generation of males

Since *C. elegans* males occur only at a low frequency (~0.02%) in wildtype hermaphrodite populations, it is important to maintain mating stocks for crosses. To get enough males to set up a mating stock, two methods were used for the experiments in this thesis. For transgenic worms, like the NL3909 and NL3908 strains (see Chapter 6) heat shock was used to gain male progeny. For this, 6 worms at the L4 stage were transferred to a freshly seeded plated and incubated at 30 °C for 6h, or 3-4h at 34°C (Sulston and Hodgkin, 1988).

In case of wildtype males, the male stock N2 DRM obtained from the CGC was used or the mentioned heat shock was performed. Once enough males were obtained a mating stock was set up. Here, L4 male and hermaphrodite worms in a 3:1 ratio were transferred with a generous amount of OP50 bacteria on the worm pick to an unseeded plate. The low amount of food concentrated in one area increased the chances of a successful cross.

The males obtained here were used for the crosses. Once a male stock was successfully set up, it was maintained by frequently placing L4 males and hermaphrodites in a 3:1 ratio on a fresh plate as described.

2.2.11.3. Crosses

Crosses are a basic genetic method and convenient tool to map mutations (see Chapter 5), to test mutations in different mutant backgrounds and to remove unwanted mutations caused by mutagenesis transgene integration (see Chapter 5) or genetic background effects (see Chapter 6).

Generally a cross is set up with hermaphrodites and males in a 1:3 ratio, respectively. How to start a cross, if the hermaphrodites should carry the mutation or the males, depends on the mutation, if it is on the X chromosome, which phenotypic marker it has or if it is lethal in homozygote state. That a cross was successful is visible in the 50% males in the F_1 generation.

2.2.11.4. Mapping

2.2.11.4.1. Two-point mapping

Various phenotypic markers on all six chromosomes are available for mapping. The first step of mapping is two-point mapping. Here the mutation of interest is mapped against marker mutations on each chromosome; therefore this step is used to determine the individual chromosome carrying the mutation (see Figure 2.2).

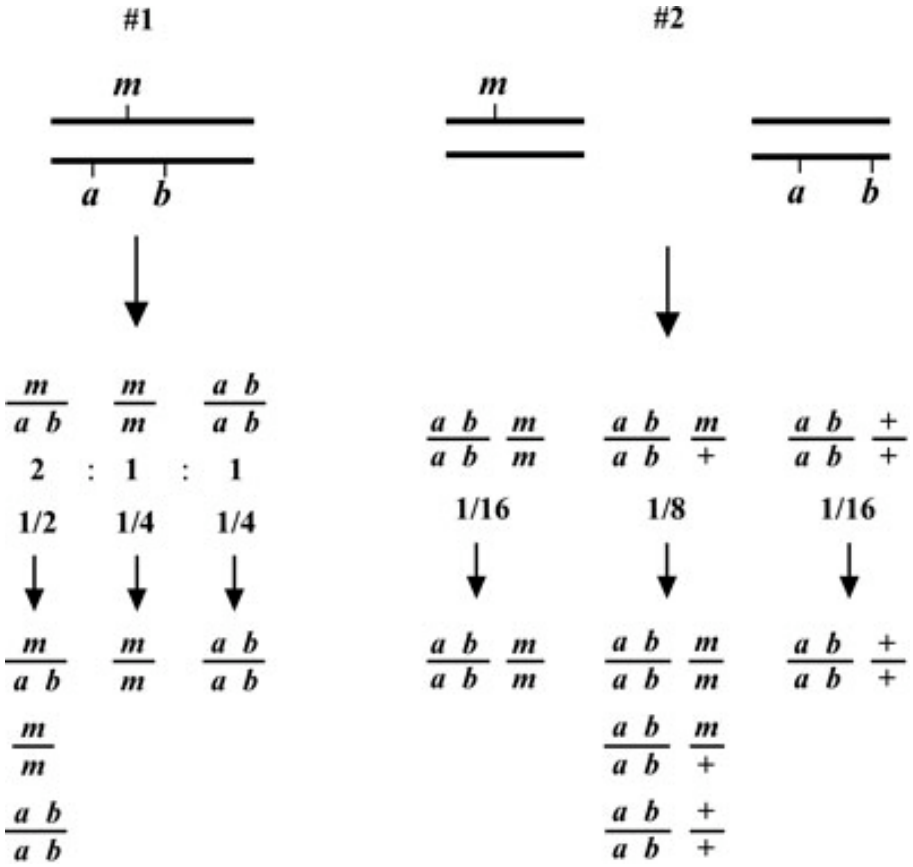


Figure 2.2: Two-point mapping. Segregation scheme if the mutation m is located on the same chromosome as the phenotypical marker (#1), or on a different one (#2) (Reproduced from WormBook (Fay, 2006a).

Concentrating on the genotypes and their frequency of the offspring, in #1 the genotypes *m*/*a b*, *m*/*m* and *a b*/*a b* have the corresponding phenotypes wildtype, M and A B. The triple mutant MAB would require a really rare recombination event. Another way to identify the triple mutant masked by the wildtype phenotype is through its offspring. The offspring of its F_1 generation with the wildtype phenotype would segregate both M and A B animals, whereas M and A B animals won't.

If the mutation is on a different chromosome than the marker, as seen in #2, 1/16 of the F_1 generation would carry the MAB phenotype, whereas 1/16 would show the A B phenotype and 1/8 will be A B and heterozygote for M. However, this segregation scheme is also possible if marker and mutation are located at the ends the same chromosome.

2.2.11.4.2. Three-point mapping

Three-point mapping is used to determine the exact location of the mutation on the identified chromosome. Therefore a mapping strain with two phenotypical markers A and B are crossed with our mutation M. The segregation pattern in the F_2 generation shows where M is located (see Figure 2.3).

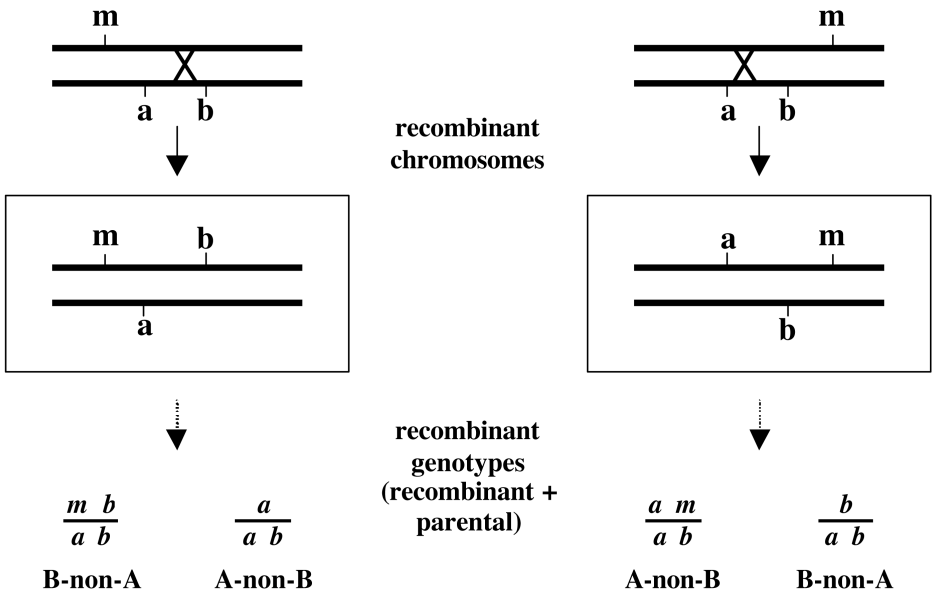


Figure 2.3: Three-point mapping with *m* to the left or right. Scheme if M lies to the right of left of the markers (Reproduced from WormBook (Fay, 2006a).

When *m* is located to the left, all B-non-A offspring will segregate B M progeny, as well as B and A B segregants. On the other hand, A-non-B segregants will segregate only A and A B progeny. This kind of segregation indicates that *m* lies to the left of *a* since the chances for a crossing over in close proximity are small. If *m* lies to the right of *b*, the A-non-B recombinants will have A M offspring, whereas the B-non-A ones will segregate B and A B animals. This is the fist step of three-point mapping. However, this step does not give any information about the distance to the left or right of the appointed marker the mutation is. To determine the exact location, it is necessary to use a mapping strain with two phenotypic markers that flank the mutation. In this scenario the mutation *m* lies between *a* and *b*.

The distance separating two genes on the same chromosome can be determined by the frequency of meiotic recombinations occurring between them. Usually one recombination occurs between two homologous

chromosomes during meiosis. The closer the genes are located to each other, the rarer a recombination event happens between them and the farther apart the mutations are, the more likely a recombination can occur. The convention for one map unit (1.0) is describes as 1% of meiotic recombination frequency.

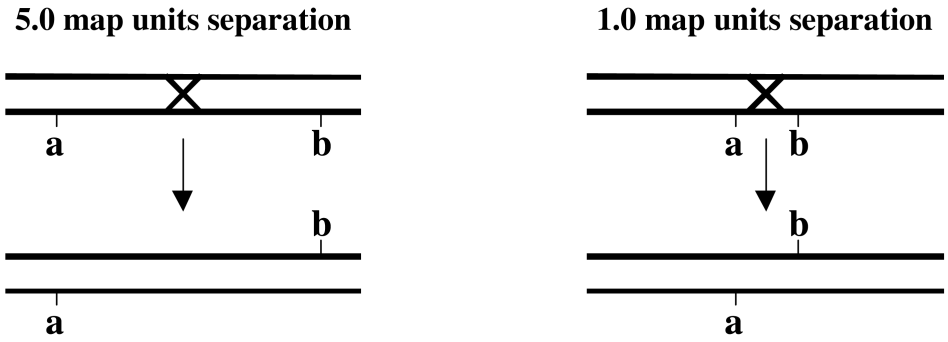


Figure 2.4: Map units convention. Example of a recombination event of two mutations five or one map unit apart from each other (Reproduced from WormBook (Fay, 2006b).

The recombination event to the left shows two mutations that are five map units apart. Therefore a recombination event would occur in 5% of the gametes. The scheme on the right shows two mutations that are one map unit apart, therefore this recombination event occurs in only 1% of the gametes. Both recombination events cause the two mutations to be genetically unlinked. Important to know is that two mutations that are located at the opposite ends of the same chromosome (50 map units apart) have a 50% chance to be separated during meiosis and will therefore also seem to be genetically unlinked. Coming back to mapping, crossing a mutant *m* with a strain that has two phenotypical markers *a* and *b* flanking *m*, the following scenarios are possible:

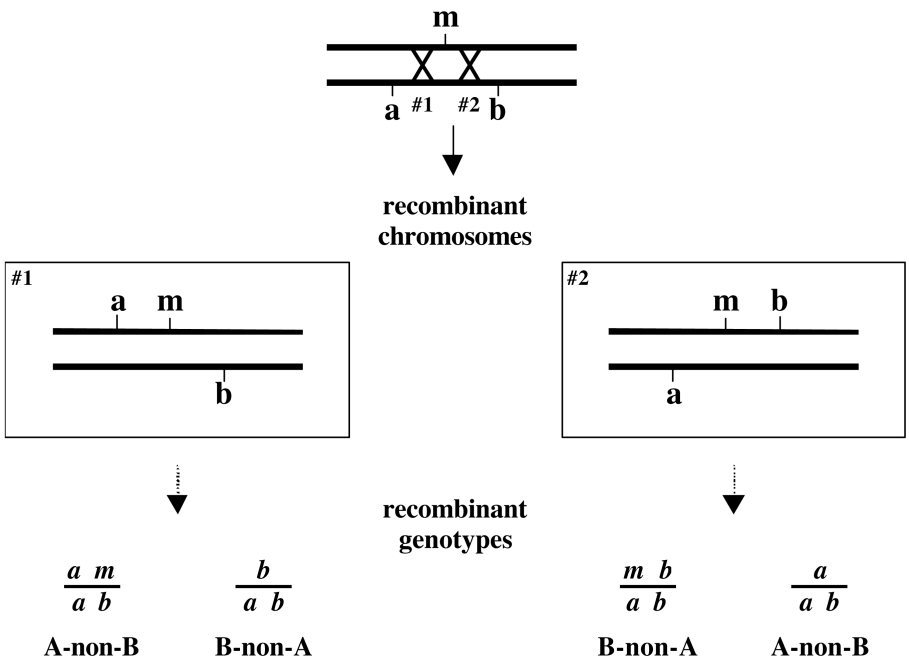


Figure 2.5: Three-point mapping with m in the middle. Here, the mutation is flanked by the two markers (Reproduced from WormBook (Fay, 2006b).

The possible recombination events and the resulting segregants are shown in Figure 2.5. Here the ratio of recombinants is the determining factor to locate the map position of *m*. The closer *m* is to *a*/*b*, the lower is the recombination frequency with this marker. The recombination frequency reveals the location of *m* on the chromosome respectively to the two markers used (see Figure 2.5).

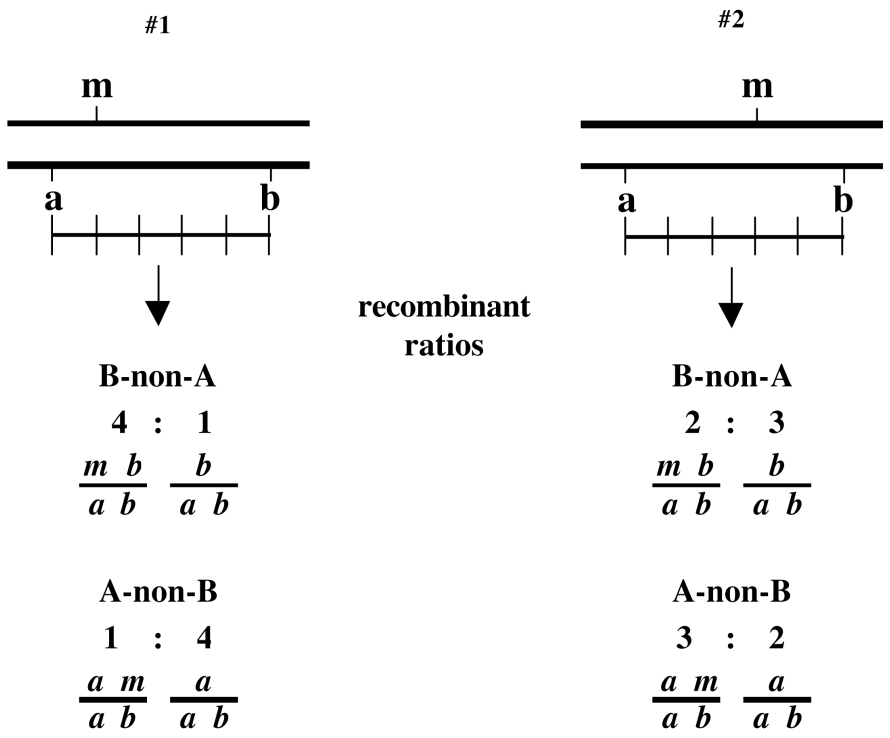


Figure 2.6: Map position calculations. Three-point mapping to determine the exact position of a mutation (Reproduced from WormBook (Fay, 2006b).

In the scenario #1 of Figure 2.6, the mutation *m* is only one map unit away from *a*, whereas it is four map units away from *b*. Therefore B-non-A segregants will have a higher frequency of m recombinants (4:1) than A-non-B animals. In scenario #2, *m* is two map units apart from *b* and three from *a*. Therefore B-non-A segregants will show m recombinants in a ratio of 2:3, whereas A-non-B will segregate *m* recombinants in 3:2 ratios.

2.2.12. Molecular biology

2.2.12.1. RNA extraction

1-bromo-3-chloropropane (BCP) was used in RNA extractions. BCP was used as a replacement for chloroform, which is a highly volatile and toxic reagent. Using BCP instead of chloroform reduced the handling of toxic material and decreased the possibility of contaminating RNA with DNA, without any adverse effects on the quality of isolated RNA as described in a standard protocol (Chomczynski and Mackey, 1995).

The worms were collected by washing at least two big plates (100 mm) with M9 buffer and gathering all the worms in a 200 μl worm pellet. Then the worm pellets were transferred to tubes of Lysing Matrix D (MP Bio) pre-filled with 800 μl Trizol and frozen immediately in liquid nitrogen. At this step the samples could be stored at -80°C until use.

Then the samples were placed into a Ribolyser (Hybaid) and homogenized in the lysing matrix tube. Homogenization was carried out at the setting 6.5 for two periods of 10 seconds. The tubes were incubated for 5 min at room temperature before adding 200 μl of BCP and vortexing them for 15 seconds. Then an incubation for 3 minutes at room temperature and centrifugation at 4°C for 15 minutes at 12000rpm followed to separate the phases. Meanwhile sterile RNAse free Eppendorf tubes were prepared on ice, in which the aqueous upper layer of the samples were transferred. Then 600 μl of isopropanol and 1 μl glycogen blue were added and carefully mixed by inverting the tubes. Afterwards incubation for 10 min at room temperature was used to precipitate the RNA. A centrifugation at 4°C for 10 minutes at

14000rpm followed to recover the RNA. The supernatant was carefully removed from the pellet. 100 μl ethanol was added to the samples and vortexed briefly to wash the pellets, and spun at 4°C for 5 minutes at 7500 rpm. The supernatant was removed carefully and the pellet air-dried for 10 minutes. Afterwards the pellets was dissolved in 50 μl sterile H_2O by incubating at 60°C for 10 minutes. To determine the RNA concentration and purity, each sample was diluted 1:1000 and the OD at 260/280 taken using a Eppendorf Bio Photometer.

2.2.12.2. First strand cDNA synthesis

To create a cDNA template from RNA, the Invitrogen Superscript II kit was used. An RNA concentration of 2 μM was used. RNA and H_2O were mixed to a final volume of 10 μl and 1 μl of oligo dT and 1μl of a 10mM dNTP were added and mixed. The samples were incubated at 65°C for 5 min and then chilled on ice. After a short centrifugation, 4 μl of 5x First-Strand buffer, 2 μl of 0.1 M DTT and 1 μl of RNaseOUT recombinant ribonuclease inhibitor were added. This was gently mixed by adverting the tube before 1 μl of SUPERSCRIPT II was added. The remaining steps were done in a PCR machine with the cycles 42°C for 52 min and 70°C for 15 min. Afterwards the cDNA was ready to be used as a template in PCRs.

2.2.12.3. Real-Time (RT) polymerase chain reaction (PCR)

RT-PCR is a technique based on polymerase chain reaction. It enables detection and quantification of a specific sequence in a DNA sample. Its key feature is that the amplified DNA is quantified as it accumulates in the reaction after each amplification cycle. To quantify the mRNA level of a specific gene, such as *ftn-1* or *sir-2.1* in the different transgenic lines (see Chapter 4 or 5), an RT-PCR was performed. The first steps were RNA extraction and a reverse transcription. For RT-PCR reaction the ABI SYBER Green PCR mix and the following primers were used (*ftn-1* primers [Chapter 3] and *sir-2.1* primers [Chapter 5]).

sir-2.1_fwd2	AGAACGCGCATTTCGCCATATTAAG
sir-2.1_rev2	ATACTGACACTCCAGCGCCAG
ftn-1_fwd_RT2	CGGCCGTCAATAAACAGATTAACG
ftn-1_rev_RT2	CACGCTCCTCATCCGATTGC

Table 2.2: RT-PCR reaction.

Component	Amount (µl)
SYBER Green 2x PCR mix	12.5
H_2O	10.5
Primer mix (µM)	1
cDNA	1

The ABI 7000 machine was used to run my samples.

The quantification was conducted according the delta-delta C_t method, comparing the samples of interest with the appropriate controls, which were normalized to the endogenous housekeeping gene *ama-1*.

2.2.13. Biochemistry

2.2.13.1. Protein gels

Components were pipetted according the order of the list presented below to two 50 ml Falcon tube.

Table 2.3: Components for Western blot gel

Components	2 gels 12%	2 upper gels 3%
H_2O	8.4 ml	5 ml
Tris 1.5 M pH 8.8 (upper gel 0.5 Tris pH 0.8)	4.92 ml	3.74 ml
SDS 10%	196.8 µl	150 µl
Acrylamide (40%)	6 ml	1200 µl
Ammonium persulfate 10%	225 µl	300 µl
TEMED	15 µl	20 µl

The contents were mixed by inverting the Falcon tube several times. TEMED (tetramethylethylene) was then added and the contents mixed again. 11 ml of the mix were transferred to a western blot cassette by pressing the pipette tip against cassette wall and letting the mix slowly fill the cassette. To get rid of bubbles on the surface, isopropanol was added. After 1-2 h incubation, isopropanol was removed and the upper layer washed with sterile H_2O. Blotter paper was used to take off excess liquid. The contents for the 3% gel preparation were mixed in as described before. The 3% gel mix solution was transferred into the gel cassette until the surface reached the end of the comb marks. To avoid air bubbles, the comb was tilted and carefully pressed into position. Again the excess liquid was removed with blotter paper. After

one hour, the gel was ready for use. If 1 x transfer buffer is added to the top of the cassette, gels can be prepared one or two days in advance and stored at 4°C.

2.2.13.2. Protein sample collection

Synchronized worms were washed off plates using M9 buffer (Worms can be synchronized by doing an egg prep and leaving the eggs to hatch on a plate overnight at 20°C on M9 without food. Worms will arrest as L1s). The worms were transferred with a glass pipette from Falcon tubes to Eppendorf microcentrifuge tubes and washed three times with M9 buffer. After the last washing step, as much supernatant as possible was removed without disturbing the worm pellet and the sample was frozen at -20°C until use.

2.2.13.3. Protein extraction

Samples prepared as described in section 2.2.13.2. above were thawed and centrifuged shortly to collect the worms in a pellet. The supernatant was removed and 100-200 µl of protein buffer was added to the worm pellet. The sample was thawed and frozen three times at – 80ºC (optional). Alternative or additionally each sample was sonicated six times for 10 sec with the sonicator until the worm pellet was completely fragmented. In between the sonication steps, the tubes were kept on ice. A 30 min centrifugation followed at 14000rpm at 4°C. Afterwards, a clear phase and white phase appeared. The supernatant with both phases was transferred to new Eppendorf microcentrifuge tubes and briefly vortexed. The protein

concentration was determined using the Bradford assay. A protein concentration of at least 15-20 µg/µl was necessary for western blotting.

2.2.13.4. Preparation of oxyblot samples

Oxyblots were performed in the similar way to standard Western blots e.g. in terms of the protein sample preparation after thawing and determination of the protein concentration. After the latter step, the necessary volumes of protein were transferred to Eppendorf tubes. The volume of each sample was adjusted to 5 µl by adding H_2O. Then 5 µl of 12% SDS was added to the samples to denature the proteins. 10 µl of 1x dinitrophenylhydrazine (DNPH) (dilute 1:10) was added and incubated for 15 min at room temperature (in case of using the negative control, 10 µl of the 1 x derivatization-control solution was added instead of the DNPH solution). After the incubation step, 7.5 µl of neutral solution was added to stop the reaction. At this point, the samples could be stored at 4ºC for up to one week.

2.2.13.5. Western blotting

In the case of a Western blot, loading buffer in a 5 x concentration was added to each sample before transferring it to the gel. Oxyblot samples already contained a loading dye in the neutral solution. A pre-strained molecular weight marker was transferred to the first and last well in the gel. The gels were run at 200 V for 1 h until the dye front had migrated to the bottom of the gel. The transfers were performed at 100 V for 45min. The membranes were washed in blocking solution for 1 h before the incubation of

the primary antibody followed at 4°C overnight. The next day three 20 min wash steps with PBS-Tween followed before a one hour incubation with the secondary antibody. The membrane was then washed three times for 30 min with PBS-Tween. The blots were analyzed using chemiluminescent reagents according to manufacturer's directions (ECL Plus Western Blotting Detection System, Amersham).

To detect the actin protein concentration, which was used as an internal control, the membrane was washed with the Restore Plus Western Blot Stripping Buffer according manufacturer's directions (Thermo Scientific). Incubation of first and secondary antibody was performed as described.

2.2.14. Measuring free iron levels using electron paramagnetic resonance (EPR) spectroscopy

2.2.14.1. The theory behind the method

EPR spectroscopy, also called electron spin resonance (ESR) or electron magnetic resonance (EMR) is basically the resonance spectroscopy of molecular systems with unpaired electrons.

EPR uses microwave radiation to detect unpaired electrons and the magnetic field can be set at *g*=4.3. At this *g* only high spin rhombic ferric iron can be detected, because ferrous iron signals are too broad to be detected. This fact makes it also possible to distinguish between the free iron levels and iron attached to proteins or Fe-S clusters. These irons are detectable at different *g* values (Pate et al., 2006). This method showed increased free iron

levels in SOD knockout strains of *E. coli* and yeast (Keyer and Imlay, 1996; Srinivasan et al., 2000).

2.2.14.2. Sample preparation

Four-six 10cm plates of synchronized worms were used to prepare the sample. The worms were washed off the plates using M9 buffer. The sample was washed three times with M9 buffer and centrifuged for 3 min at 4°C at 5000 rpm. The supernatant was removed and the worm pellet was resuspended in 15% glycerol. To detect also the free iron in ferrous state, DF was added to the samples using a final concentration of 2 mM and incubated for 15 min at room temperature. During the incubation time, the samples were transferred into the EPR tubes using a 30 cm long glass pipette to ensure the worms are mainly collected at the bottom of the tube and not along the tube walls. After the incubation time, the tubes were transferred into ice to speed up the segregation of the worms to the bottom of the tube. After all the worms were settled at the bottom, as much as possible of the supernatant was removed and the samples were flash frozen in liquid nitrogen and stored at -80°C or in a liquid nitrogen tank until further use (Pate et al., 2006).

2.2.14.3. EPR spectroscopy

The study in 2006 counted the worms in each sample and used iron standards to measure the exact iron concentration within the worms (Pate et al., 2006). We standardized our sample using the manganese signal, since we were mostly interested on the effect of iron treatment or *ftn-1* over-expression on the free iron levels in *C. elegans*.

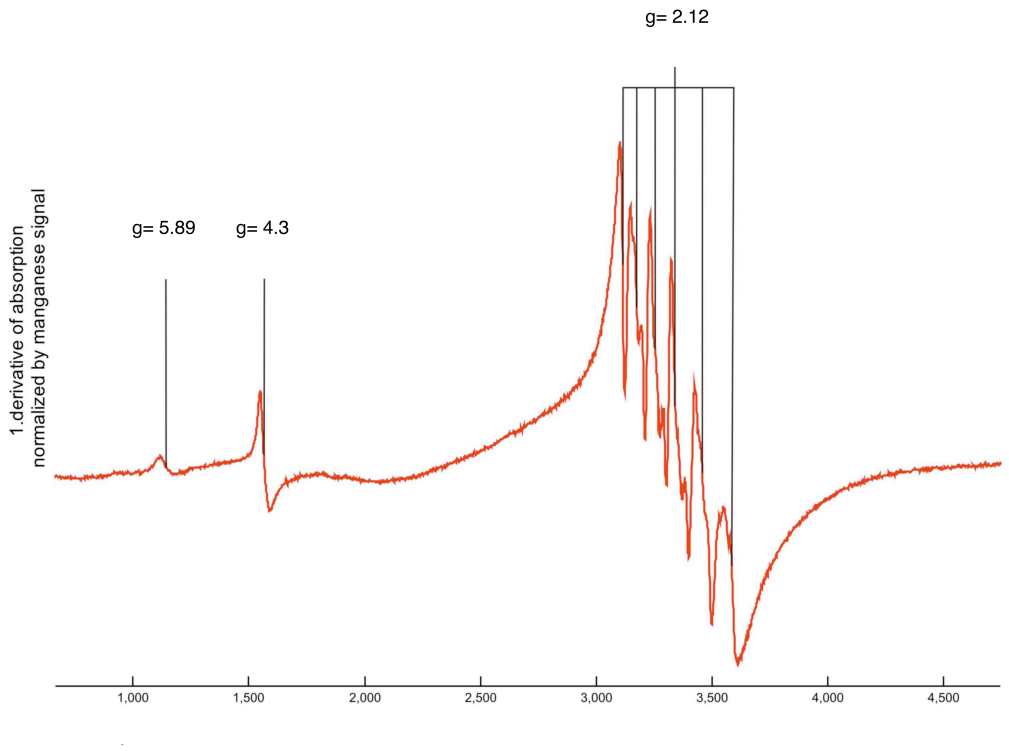


Figure 2.7: EPR spectra of wildtype worms. The EPR spectra detected a heme signal at *g*=5.89, an iron (III) signal at *g*=4.3 and a manganese signal at *g*=2.12. The manganese signal was used to standardize all the samples.

The measurements were performed with a Bruker EMX machine with super high Q resonator and cryostat for low temperature measurements at 10K. During measurements, the parameters were set at: microwave setting at 9.5 GHz at X-band, center field at 3000 Gauss, sweep with at 6000 Gauss, microwave power at 2mV, modulation amplitude 10 Gauss and modulation frequency at 100 kHz. The iron peak was detected at g=4.3 and a lock-in detection helped to increase of the signal relative to background noise.

Chapter 3: Investigating the role of oxidative damage in *C. elegans* ageing

3.1. Investigating the role of ferritin in *daf-2* longevity

3.1.1. Why are we interested in *daf-2* mutants?

daf-2 mutants, defective in the insulin/IGF receptor, are long-lived and therefore intensively studied in the *C. elegans* ageing field (see Introduction, section 1.4.7.). What might cause a small soil nematode to double its mean life span? To what extend are these determinants of ageing conserved across the animal kingdom? These questions have occupied scientists in the ageing field for a number of years.

Is it possible to slow ageing in humans? Biogerontologists are not only interested in increasing the maximum life span, but also improving health and quality of life in old age. *daf-2* mutants are generally more active even at advanced ages and more resistant against various stresses including heat stress, UV and ROS (see Introduction, section 1.4.7.). The resistance of long-lived insulin/IGF signaling (IIS) mutants to ROS is consistent with the oxidative damage theory, which sees ageing as a result of accumulated oxidative damage over time (see Introduction, section 1.2.2.1.). The theory predicts that in mutants with extended life span one might see reduced ROS production and/or an increased ability to detoxify ROS. The latter prediction is supported by various studies showing elevated levels of antioxidant enzymes, such as SOD and catalase in long-lived *daf-2* and *age-1* mutants (Larsen, 1993; Vanfleteren, 1993; Vanfleteren and De Vreese, 1995).

3.1.2. The discovery of elevated *ftn-1* levels in *daf-2* mutants

In 2004 a microarray screen identified a large number of genes with altered gene expression in *daf-2* mutants in a *daf-16*-dependent manner, and suggested up to 10% of the genes in *C. elegans* genome are regulated by *daf-2* and *daf-16*. These include possible genetic determinants of ageing (McElwee et al., 2004). Some of the differentially expressed genes were associated with metabolism, stress response, and the dauer stage. One of the up-regulated stress response genes encoded the iron storage protein ferritin 1 (*ftn-1)* (see Introduction, section 1.2.2.5.2.). The microarray data revealed a 47.14 fold increase in *ftn-1* levels in *daf-2* mutants relative to *daf-16; daf-2* double mutants. This is the fourth largest increase in gene expression detected in *daf-2* mutants (the highest was *scl-20,* D1086.9 and B0238.12) (McElwee et al., 2007; McElwee et al., 2004). *ftn-1* is also 6.44-fold up-regulated in worms recovered from dauer stage (Wang and Kim, 2003). The other ferritin gene, *ftn-2*, is not up-regulated. My colleague, Dr Daniel Ackerman, created a transgenic line with the green fluorescent protein (GFP) expressed from an *ftn-1* promoter. *Pftn-1::GFP* was strongly induced by mutation of *daf-2*, confirming the microarray results (Ackerman, 2010).

Considering the role of iron in oxidative damage (see Introduction, section 1.2.2.4.) the up-regulation of *ftn-1* in *daf-2* mutants and dauer larvae could imply a role for iron homeostasis in longevity assurance.

3.1.3. Aims

The aims of work in this chapter are to test the role of *ftn-1* in *daf-2* longevity. This contributes to a broader aim of testing the importance of iron homeostasis in longevity assurance. I test the importance of *ftn-1* in the extended life span of *daf-2* mutants, and oxidative stress resistance here. For this I used *ftn-1* RNAi in *daf-2* mutants.

Furthermore, I explored whether *ftn-1* protects against ageing by reducing levels of free iron that would otherwise cause oxidative damage via the Fenton reaction. To this end, I performed a series of manipulations of iron and *ftn-1* expression levels in *C. elegans* and studied their effects. I tested whether increased free iron levels caused by iron administration led to increased oxidative damage levels and accelerated ageing. I also tested whether reduced free iron levels, using the iron chelator deferoxamine (DF) increased *C. elegans* life span.

3.2. Results

3.2.1. *ftn-1* does not contribute to *daf-2* mutant longevity

To test whether the elevated *ftn-1* levels in *daf-2* mutants contribute to their longevity, I reduced *ftn-1* expression using RNAi and observed the effect on life span. I used the *daf-2* mutant GA303 (*rrf-3(pk1426) II; daf-2(m577) III*) and as a control the strain NL2099 (*rrf-3(pk1426) II)*. The *rrf-3* gene encodes an RNA-directed polymerase homolog and mutation of this gene results in hypersensitivity to RNAi. *daf-16* RNAi and the empty RNAi feeding clone L4440 were included as positive and negative controls, respectively. The bacteria containing the L4440 plasmid are identical to the bacteria used for the *ftn-1* RNAi, but without the *ftn-1* fragment inserted into the plasmid. DAF-16 is a forkhead box O (FOXO) homologue that acts in the IIS pathway and regulates dauer formation, stress response, fat metabolism and longevity in *C. elegans*. Mutations or reduced expression levels of this gene result in stress sensitive and short-lived animals.

Prior to life span measurements, I kept the animals on the RNAi bacteria for two generations to maximize the effect of RNAi. To test the effectiveness of the RNAi in reducing the *ftn-1* expression levels in *daf-2* mutants, I used real-time (RT) PCR (see Materials & Methods, section 2.2.12.3.). For simplicity's sake, in the sections *daf-2* mutants are denoted *daf-2(-)* and the strain wildtype for *daf-2* are denoted *daf-2(+)*. The results are presented in Figure 3.1 and Table 3.1.

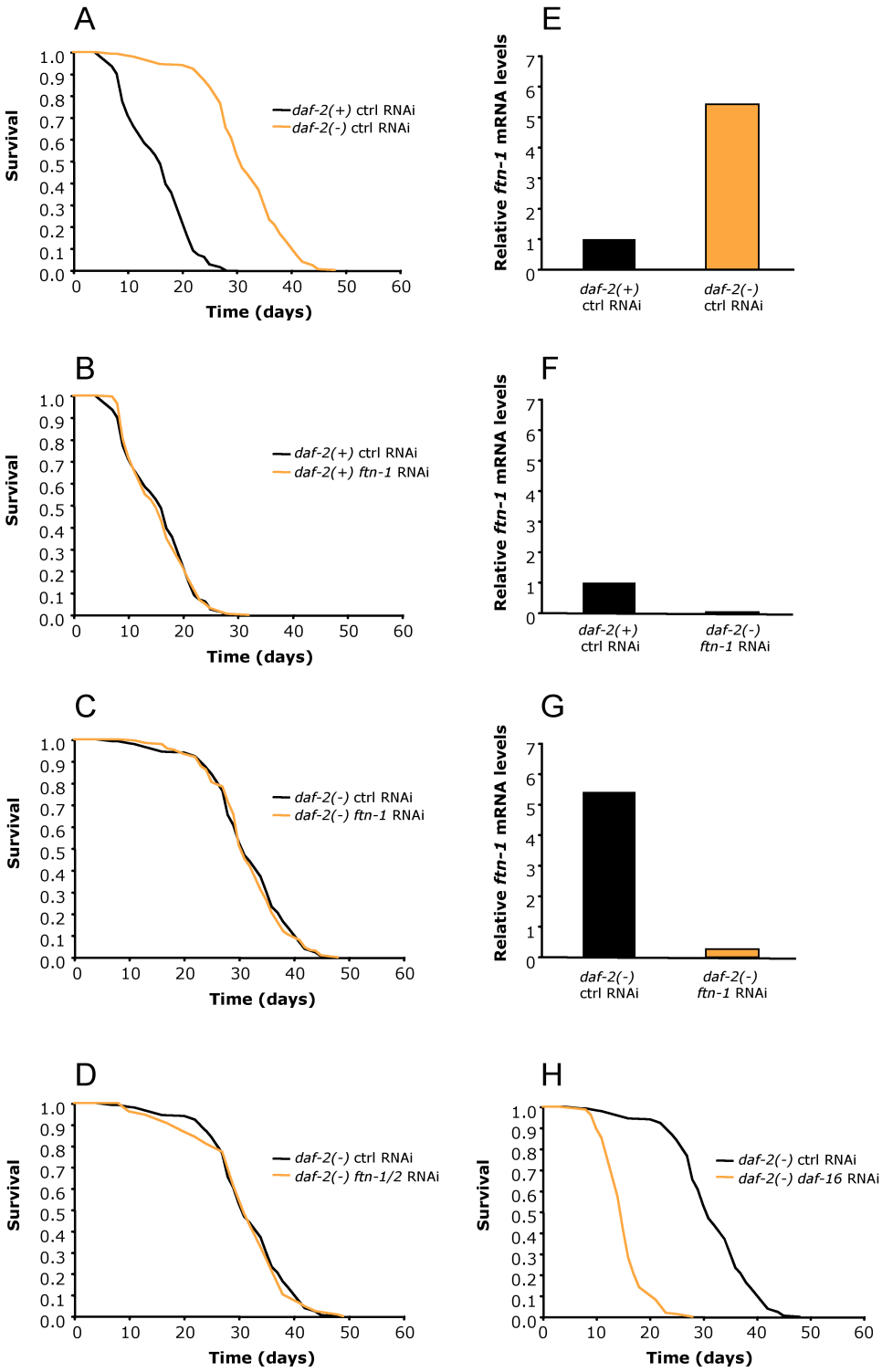


Figure 3.1: Effect of *ftn-1* RNAi on life span in *daf-2* mutants. Genotypes of *daf-2(+)* and *daf-2(-)*: NL2099 (*rrf-3(pk1426) II),* GA303 (*rrf-3(pk1426) II; daf-2(m577) III),* respectively. **(A-D, H)** Life span curves of *daf-2(+)* and *daf-2(-)* on control and *ftn-1* RNAi. Life span data represents the combined data of two independent biological replicates on control (ctrl) and *ftn-1* RNAi and a single trial with the combined *ftn-1* and *ftn-2* feeding clone. The statistical analysis is presented in Table 3.1. **(E-G)** The graphs show the corresponding *ftn-1* mRNA levels of each strain on control and *ftn-1* RNAi using RT-PCR. Each column represent the mean of two biological replicates, therefore statistical analysis could not be conducted.

Table 3.1: Effect of *ftn-1* RNAi on life span in *daf-2* mutants. The trials were performed at 25˚C, without FUdR. Ctrl RNAi is the L4440 plasmid vector. [C] combined data. Genotypes of *daf-2(+)* and *daf-2(-)*: NL2099 (*rrf-3(pk1426) II),* GA303 (*rrf-3(pk1426) II; daf-2(m577) III)* respectively*, p*, probability of being the same as specified control (log rank). [n] Independent biological replicate number.

Strain	RNAi	Deaths/ censored	Mean life span (days)	% *vs. daf-2(+)* ctrl RNAi	*p vs. daf-2(+)* ctrl RNAi	% *vs. daf-2(-)* ctrl RNAi	*p vs. daf-2(-)* ctrl RNAi
daf-2(+)	Ctrl	**[C] 232/18**	**15.6**				
		[1] 112/13	14.5				
		[2] 120/5	16.5				
daf-2(+)	*ftn-1*	**[C] 233/17**	**15.5**	**-0.6**	**0.98**		
		[1] 116/9	15.5	+6.9	0.19		
		[2] 117/8	15.4	-6.7	0.07		
daf-2(+)	*daf-16*	**[C] 172/78**	**12.8**	**-17.9**	**<.0001**		
		[1] 80/45	12.7	-12.4	0.0007		
		[2] 92/33	12.9	-21.8	<.0001		
daf-2(+)	*ftn-1/ftn-2*	[2] 119/7	15.7	-4.8	0.12		
daf-2(-)	Ctrl	**[C] 233/17**	**31.5**	**+101.9**	**<.0001**		
		[1] 114/11	29.9	+106.2	<.0001		
		[2] 119/6	33	+100	<.0001		
daf-2(-)	*ftn-1*	**[C] 241/13**	**31.4**	**+101.3**	**<.0001**	**-0.3**	**0.72**
		[1] 121/4	30.7	+111.7	<.0001	+2.7	0.79
		[2] 120/5	32	+93.9	<.0001	-3	0.53
daf-2(-)	*daf-16*	**[C] 226/25**	**15.3**	**-1.9**	**0.06**	**-51.4**	**<.0001**
		[1] 114/12	17	+17.2	0.10	-43.2	<.0001
		[2] 112/13	13.7	-17	<.0001	-58.5	<.0001
daf-2(-)	*ftn-1/ftn-2*	[2] 127/5	31.7	+92.1	<.0001	-4	0.26

RT-PCR showed a 95.2% reduction of *ftn-1* expression in *daf-2* mutants and a 97% reduction in control animals upon *ftn-1* RNAi, confirming the effectiveness of the treatment. Further confirmation was provided by the reduction in life span on *daf-16* RNAi compared to control animals (*daf-2(+)*: 12.7, 12.9 days *vs.* 14.5, 16.5 days, respectively, $p<.0001$ and *daf-2(-)*: 13.7, 17 *vs.* 29.9, 33, respectively, $p<.0001$, Figure 3.1, H, see Table 3.1).

Control animals showed no reduction in life span upon reduced *ftn-1* expression (15.4, 15.5 days *vs.* 14.5, 16.5 days respectively, $p=0.98$, Figure 3.1, B) and neither did *daf-2* mutants (30.7, 32 days *vs.* 29.9, 33 days respectively, $p=0.71$, Figure 3.1, C). The trial including *ftn-1* and *ftn-2* RNAi showed also no significant effect on life span of *daf-2* and control animals compared to control RNAi trials (31.5 days *vs.* 31.7 days, $p=0.26$ and 15.6 days *vs.* 15.7 days, $p=0.12$ respectively, Figure 3.1, D).

These results show that *ftn-1* is not essential for the normal life span of wildtype animals, nor does *ftn-1* up-regulation contribute to the increased longevity of *daf-2* mutants. Other possible effects of *ftn-1* up-regulation in *daf-2* mutants are investigated in the following section.

3.2.2. *ftn-1* protects against oxidative stress

daf-2 mutants showed increased resistance to oxidative stress, as induced by paraquat and menadione exposure (Honda and Honda, 1999). Menadione, like paraquat, is used to induce oxidative stress in *C. elegans* (Thor et al., 1982), and works by depleting glutathione (Chang et al., 1992). One possibility is that increased *ftn-1* expression in *daf-2* mutants contributes to this resistance. To investigate this possibility, I tested the effect of

knockdown of *ftn-1* expression levels using RNAi on oxidative stress resistance.

First I used hydrogen peroxide (H_2O_2) with the aim of mimicking the naturally occurring oxidative stress caused by the Fenton reaction, which generates hydroxyl radicals. However, the H_2O_2 assays showed large differences between individual trials (data not shown), probably because hydrogen peroxide is highly unstable, leading to differences in intensity of H_2O_2 exposure. Therefore I choose to use *tert*-Butyl hydroperoxide (TBH, $(CH_3)_3$ COOH) for the oxidative stress assays (see Materials & Methods, section 2.2.6.2. and Introduction, section 1.4.10.). Like H_2O_2, TBH can undergo the Fenton reaction, but it is a relatively stable compound.

Before starting oxidative stress assays, I maintained the *daf-2* mutants on *ftn-1* RNAi for two generations to reduce *ftn-1* expression. The animals were then transferred to the TBH plates as young adults and scored every two hours for survival (see Figure 3.2, Table 3.2).

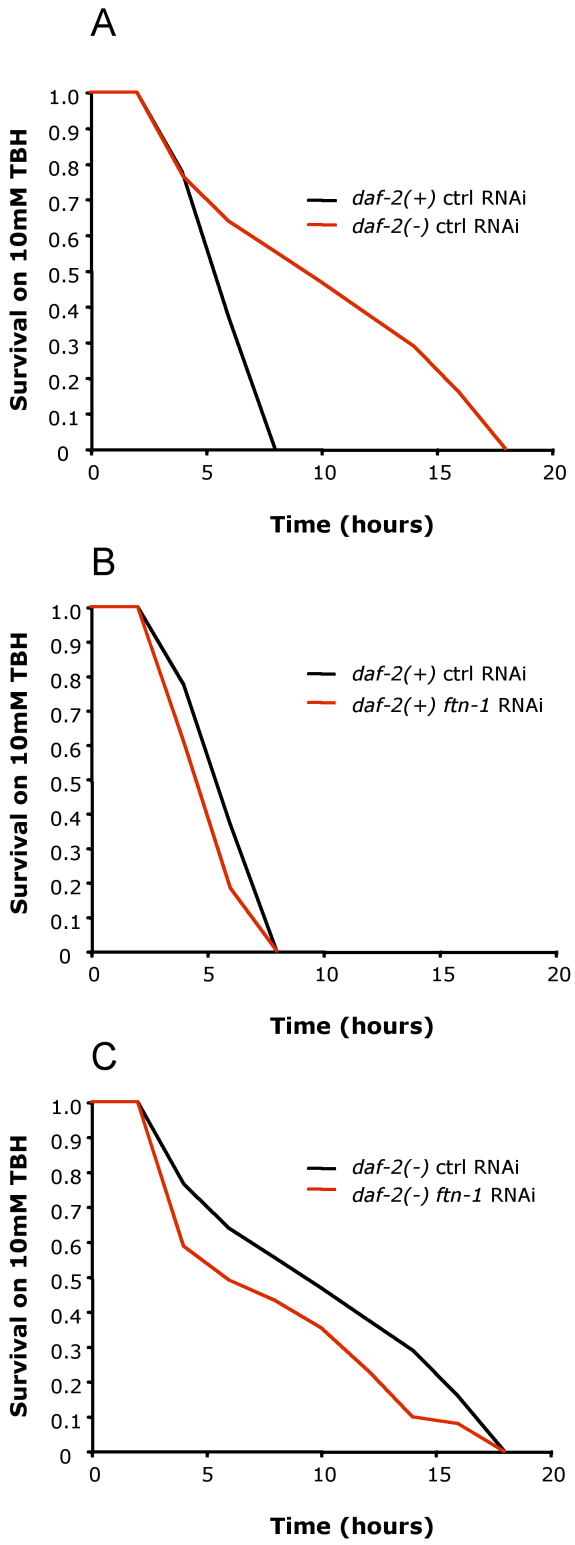


Figure 3.2: Effect of reduced *ftn-1* expression on peroxide resistance in *daf-2* mutants. Genotypes of *daf-2(+)* and *daf-2(-)*: NL2099 (*rrf-3(pk1426) II),* GA303 (*rrf-3(pk1426) II; daf-2(m577)III)*, respectively. **(A-C)** Survival curves of *daf-2(+)* and *daf-2(-)* on the control RNAi vector L4440 and *ftn-1* RNAi during increased oxidative stress induced by 10 mM TBH. Statistical analysis is presented in Table 3.2.

Table 3.2: Effect of reduced *ftn-1* expression on peroxide resistance in *daf-2* mutants. The trials were performed at room temperature on 10 mM TBH plates. Ctrl RNAi is L4440 plasmid vector. Genotypes of *daf-2(+)* and *daf-2(-)*: NL2099 (*rrf-3(pk1426) II)*, GA303 (*rrf-3(pk1426) II; daf-2(m577) III)*, respectively. *p,* log rank test.

Strain	RNAi	Deaths/ censored	Mean life span (hours)	% *vs.* genetic ctrl	*p vs.* genetic ctrl	% *vs. daf-2(+)* ctrl	*p vs. daf-2(+)* ctrl
daf-2(+)	Ctrl	52/5	6.3				
daf-2(+)	*ftn-1*	50/11	5.6	-11.1	0.01		
daf-2(-)	Ctrl	46/12	10.5			+67	<.0001
daf-2(-)	*ftn-1*	51/10	8.5	-19	0.06	+35	0.002

I confirmed the resistance of *daf-2* mutants to TBH. The IIS mutants lived 67.2% longer on 10 mM TBH compared to control animals (6.3 hours *vs.* 10.5 hours, $p<.0001$).

Reduced *ftn-1* expression significantly decreased survival of control animals on 10 mM TBH plates by 11.1% (6.3 hours *vs.* 5.6 hours, $p=0.01$). The reduction in mean survival of *daf-2* mutants was greater, 18.3%, although this difference did not quite reach statistical significance (10.5 hours *vs.* 8.5 hours, $p=0.06$). It seems likely that further trials would reveal a significant effect of reduced *ftn-1* levels on *daf-2* mutants resistance to oxidative stress.

These results imply that ferritin does protect *C. elegans* against oxidative stress induced by treatment with peroxides. This is consistent with the idea that the products of Fenton chemistry can cause increased mortality, at least where peroxide levels are high enough. However, it remains unclear whether the TBH resistance of *daf-2* mutants is in any way attributable to up-regulation of *ftn-1* expression.

3.2.3. Effect of increasing iron levels

The experiments presented in the previous section did not show any detectable role of *ftn-1* in the longevity or resistance to oxidative stress in *daf-2* mutants. The lack of effect on ageing could imply one of two things. Firstly, it could imply that manipulating *ftn-1* has too little effect on free iron levels to alter the impact of Fenton chemistry on ageing. Secondly, it could be that *ftn-1* does affect free iron and Fenton chemistry, but that neither of these have any influence on ageing. To try to distinguish these possibilities, I conducted further trials, directly manipulating iron levels *in vivo* by means of iron or iron

chelator supplementary diet. I exposed N2 wildtype worms to a series of different iron concentrations using ferric ammonium citrate (FAC) and the iron chelator deferoxamine (DF), to test the effect on internal free iron levels, *ftn-1* expression, oxidative damage levels and oxidative stress resistance. FAC and DF were used in several studies to manipulate iron level in *C. elegans* (Gourley et al., 2003; Kim et al., 2004; Romney et al., 2008).

3.2.3.1. Effect of iron supplementation on free iron levels *in vivo*

I first tested the effects of dietary iron supplementation. To verify that iron supplementation increases free iron levels, I used electron paramagnetic resonance (EPR) spectroscopy. This method was previously used to measure free iron levels in *C. elegans* (Pate et al., 2006). Only Fe (III) can be detected at *g*=4.3, making it possible to distinguish between Fe (II), protein bound and Fe-S cluster bound iron. However, Fe (II) can be detected by converting it to Fe (III) by treating the samples with the iron chelator DF. In the presence of oxygen, DF catalyzes the oxidation of Fe (II) to Fe (III), making it visible at *g*=4.3. Several studies have shown that treatment with DF does not cause release of iron from iron-containing proteins into the iron pool, at least not at the incubation times used (Keyer and Imlay, 1996; Kidane et al., 2006; Pate et al., 2006; Srinivasan et al., 2000).

In *C. elegans* the relative contribution to the free iron pool of Fe (II) and Fe (III) was determined by measurements with or without DF treatment of the EPR samples. The sample without DF treatment showed a 50% lower signal at *g*=4.3 than the sample with DF treatment (Pate et al., 2006), indicating that half of the free iron pool is in Fe (II) state and half in Fe (III). In *E. coli* the free

iron pool seems to consist only of Fe (II) since EPR signals were only detectable after treatment with DF (Keyer and Imlay, 1996). On the other hand, the iron pool in yeast seems to be in Fe (III) form, since treatment with DF did not have any effect on the EPR signal (Srinivasan et al., 2000).

For the studies described here, Mustafa Alam, a undergraduate student performed the EPR measurements under my supervision and with the help of Micha Kunze, a graduate student working with Chris Kay in the Department of Structural & Molecular Biology at UCL. We grew up large quantities of synchronized worms and transferred them as young adults to control plates or plates supplemented with 5, 15 or 50 mM iron using FAC, incubated them for one day at 20˚C and collected them for the iron measurements using EPR (see Materials & Methods, section 2.2.14.).

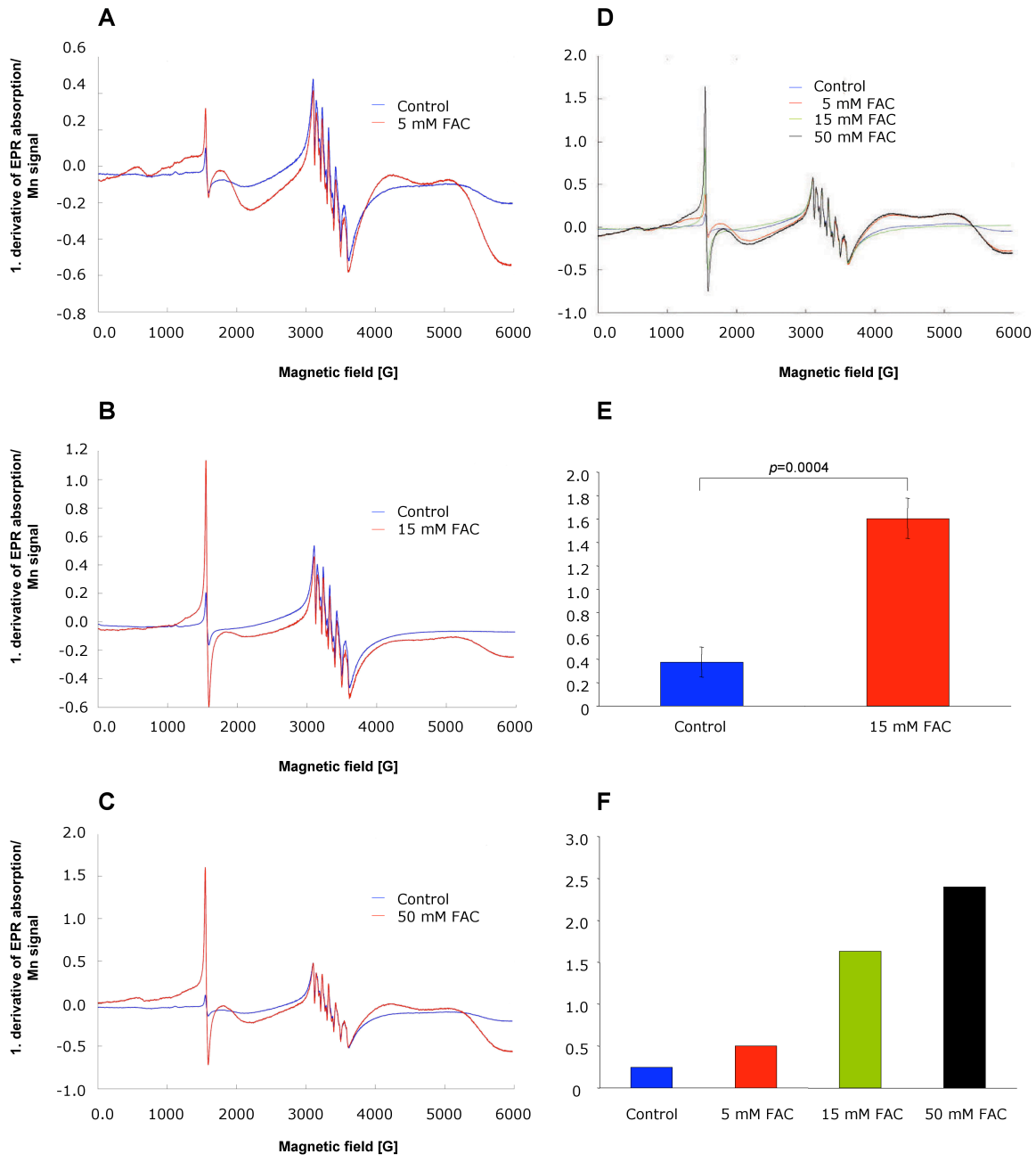


Figure 3.3: Effect of iron supplementation on free iron levels in the worm. Genotype: N2 wildtype**. (A-C)** Individual EPR measurements with control worms and ones exposed to 5, 15 and 50 mM iron. **(D)** The three iron measurements within one graph. **(E)** Each column presents the mean of three biological replicates of the measurements on 15 mM iron and their controls. Statistical analysis was performed with the Student's T-test. Error bars show the standard error of the mean (S.E.M.). **(F)** The individual iron peak lengths on the three iron concentrations of measurement [3, see Table 3.3]. It was not possible to do statistical analysis with animals on 5 and 50 mM iron plates, since the measurement at these concentrations was only performed once.

Table 3.3: Effect of iron supplementation on free iron levels in the worm. Genotype: N2 wildtype. The numbers for each EPR measurement represent the length of the iron peak. Statistical analysis was performed with the Student's T-test. It was not possible to do statistical analysis with wildtype animals on 5 mM and 50 mM iron plates, since the measurement at these concentrations was only performed once. [n] Independent biological replicate number.

Strain	**FAC (mM)**	**Size of iron peak (arbitrary units)**	**% *vs.* ctrl**	***p vs.* to ctrl**
Wildtype	Ctrl	**[C] 0.37** [1] 0.50 [2] 0.37 [3] 0.24		
Wildtype	5	[3] 0.50	+34.6	
Wildtype	15	**[C] 1.60** [1] 1.41 [2] 1.76 [3] 1.63	**+328.6** +182 +375.7 +579.9	**0.0004**
Wildtype	50	[3] 2.39	+540.7	

The EPR spectra of iron-treated worms showed a dose-dependent increase in free iron levels within the worms. The peak at 4.3 *g*, corresponding to free Fe (III), showed a significant increase in peak length (+328.6%, *p*=0.0004) on plates supplemented with a final concentration of 15 mM FAC compared to control animals. A single measurement with worms exposed to 5 mM FAC showed a 34.6% increase in peak length and 50 mM FAC an increase of 540.7% compared to control samples. However, without further trials of worms exposed to 5 mM and 50 mM iron, we cannot determine if and how significant the increase in free iron levels is. These results demonstrate that by iron supplementation controlled increases in free iron levels *in vivo* may be achieved. Although the relative contribution of Fe (II) and Fe (III) to the increase in free iron was not defined in our measurements, it seems likely that Fe (II) contributed to at least some extent, particularly given that Fe (II) supplementation was used.

3.2.3.2. Effect of increased external iron levels on *ftn-1* expression

C. elegans ftn-1 is reported to be regulated by iron and 16.5 mM and 33 mM iron have been shown to increase *ftn-1* expression 1.8 and 2-fold, respectively (Gourley et al., 2003). To test if the iron treatments used in this thesis cause an increase in *ftn-1* expression, the GFP reporter strain GA631 *lin-15(n765ts) X wuIs177 [Pftn-1::GFP, lin-15(+)],* generated by Dr Daniel Ackerman, was used. Since iron exposure within a range of 5-50 mM resulted in elevated free iron levels, I decided to test 25 mM and 50 mM for effects on *ftn-1* expression. To this end worms were raised on iron-supplemented plates and the GFP levels measured on the fifth day after egg lay using a plate-reader (see Materials & Methods, section 2.2.5.).

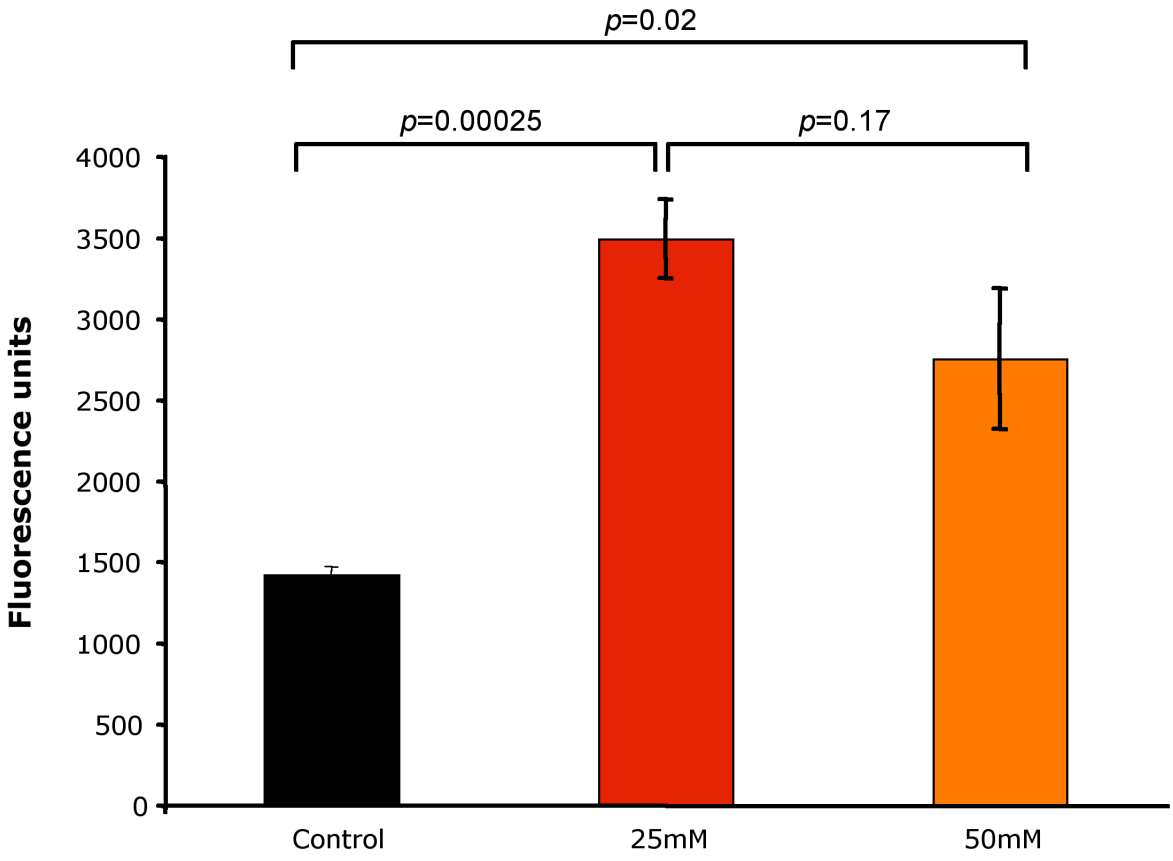


Figure 3.4: GFP expression of *Pftn-1::GFP* strain after iron supplementation. GFP expression on 25 mM and 50 mM FAC compared to control animals. The columns represent the mean of six biological replicates, the error bars show S.E.M. and statistical analysis was performed with the Student's T-test.

The *ftn-1::GFP* expression was increased by 146.6% in worms exposed to 25 mM (p=0.00025) and 94.3% in worms on 50 mM FAC (p=0.02) compared to control animals. The mean GFP expression of six biological replicates was 21.2% lower in worms exposed to 50 mM iron compared to ones exposed to 25 mM, but the difference was not significant (p=0.17).

The up-regulation of *Pftn-1::GFP* upon iron treatment is consistent with the report that *ftn-1* is up-regulated by iron in *C. elegans* (Gourley et al., 2003; Kim et al., 2004; Romney et al., 2008). The absence of a significant difference in *Pftn-1::GFP* expression between the two iron concentrations could imply that the iron concentrations used are both sufficient to maximize *ftn-1* expression, or that the bacteria on FAC supplemented plates at either concentration were iron-replete. The findings also imply that *Pftn-1::GFP* expression can act as an indicator of *in vivo* iron levels.

3.2.3.3. Iron supplementation increases oxidative damage

Having confirmed that iron supplementation elevates free iron levels within the worms, I next investigated whether this caused an increase in oxidative damage levels, as one would predict. To measure oxidative damage levels, I studied protein oxidation using the oxyblot method. This method detects carbonyl groups introduced into proteins caused by oxidative reactions, such as metal catalyzed oxidation (see Materials & Methods, section 2.2.13. and Introduction, section 1.2.2.2.). This method was previously used to measure levels of oxidized proteins in *C. elegans* (Van Raamsdonk and Hekimi, 2009; Yang et al., 2007). The level of oxidation is shown as a band in the SDS gel. Since this method labels carbonyl groups, different

individual proteins showing oxidations will appear on the gel. To detect which proteins show oxidation, mass spectrometry analysis would be necessary. Here I was not concentrating on the identity of proteins affected by the iron-catalyzed oxidative damage, but on the overall effect on protein levels.

Age synchronized worms were transferred as young adults to control plates or plates supplemented with a final concentration of 15 mM iron. These were incubated overnight and collected the next day for carbonylated protein measurement. The results are presented in Figure 3.5.

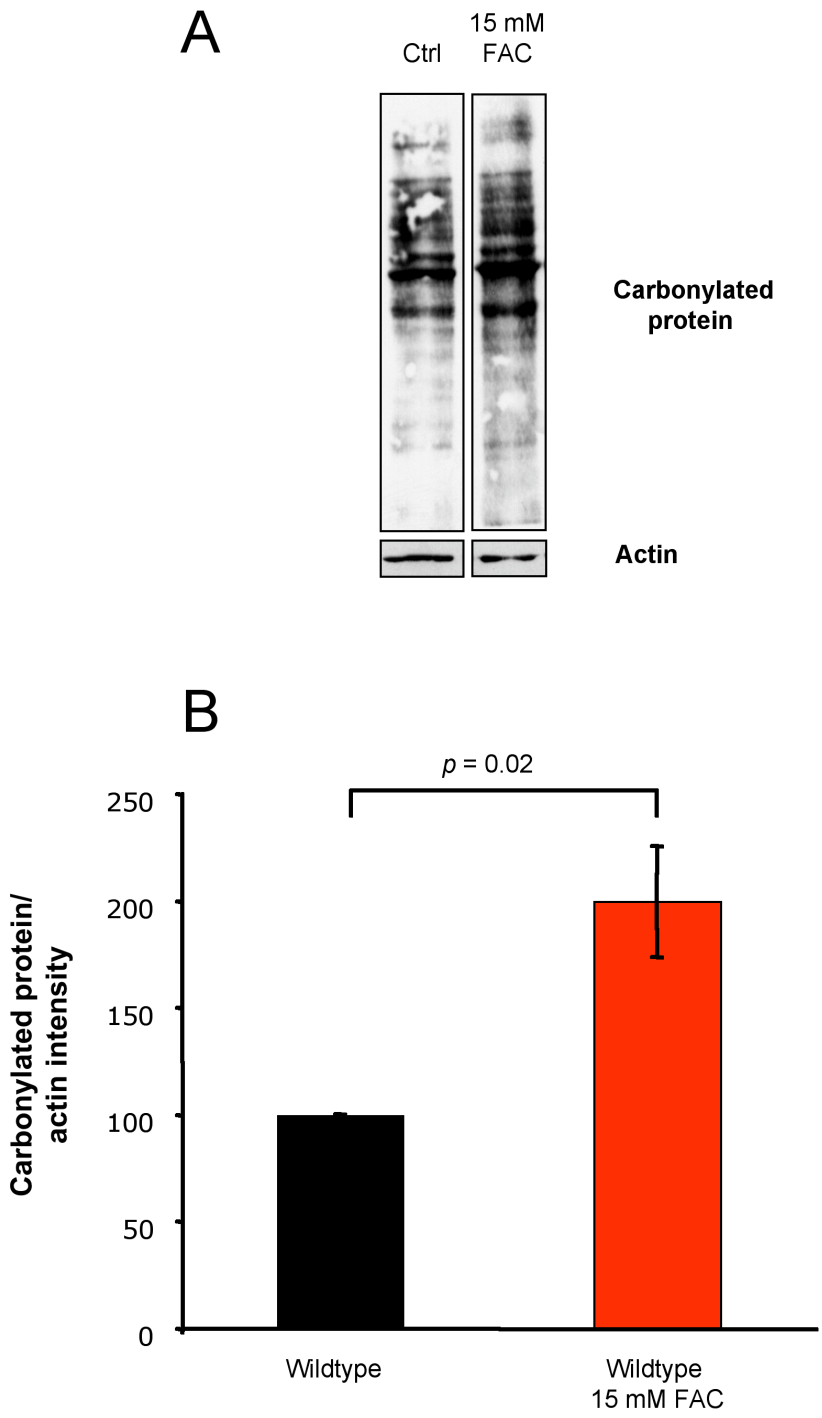


Figure 3.5: Iron supplementation increases oxidative damage in *C. elegans*. (A) A representative oxyblot showing the carbonylated protein and actin levels**. (B)** Statistical analysis of four biological replicates, two performed by Dr Filipe Cabreiro, two by myself, showing carbonylated protein levels normalized to actin immunoreactivity. The error bars show the S.E.M. and statistical analysis was performed with the Student's T-test.

Combined data of four biological replicates, two performed by Dr Filipe Cabreiro and two by myself, showed that worms exposed to 15 mM iron had 99.5% more protein carbonylation (p=0.02) compared to control animals. This result shows that iron supplementation can indeed cause increased oxidative damage in *C. elegans*.

The data presented in this section demonstrates that iron supplementation causes increased free iron levels within the worms, up-regulation of *ftn-1* expression and elevated oxidative damage levels.

3.2.4. Iron supplementation causes hypersensitivity to peroxide toxicity in *C. elegans*

Does increased free iron levels led to increased production of hydroxyl radicals via the Fenton reaction in *C. elegans*? If this were the case, then iron supplementation should increase peroxide toxicity. I therefore tested whether iron supplementation made worms hypersensitive to TBH toxicity. I exposed young adult worms for one day to a range of different iron concentrations (from 5-25 mM) and performed TBH toxicity assays on them (see Materials & Methods, section 2.2.6.2.). The results are presented in Figure 3.6 and Table 3.4.

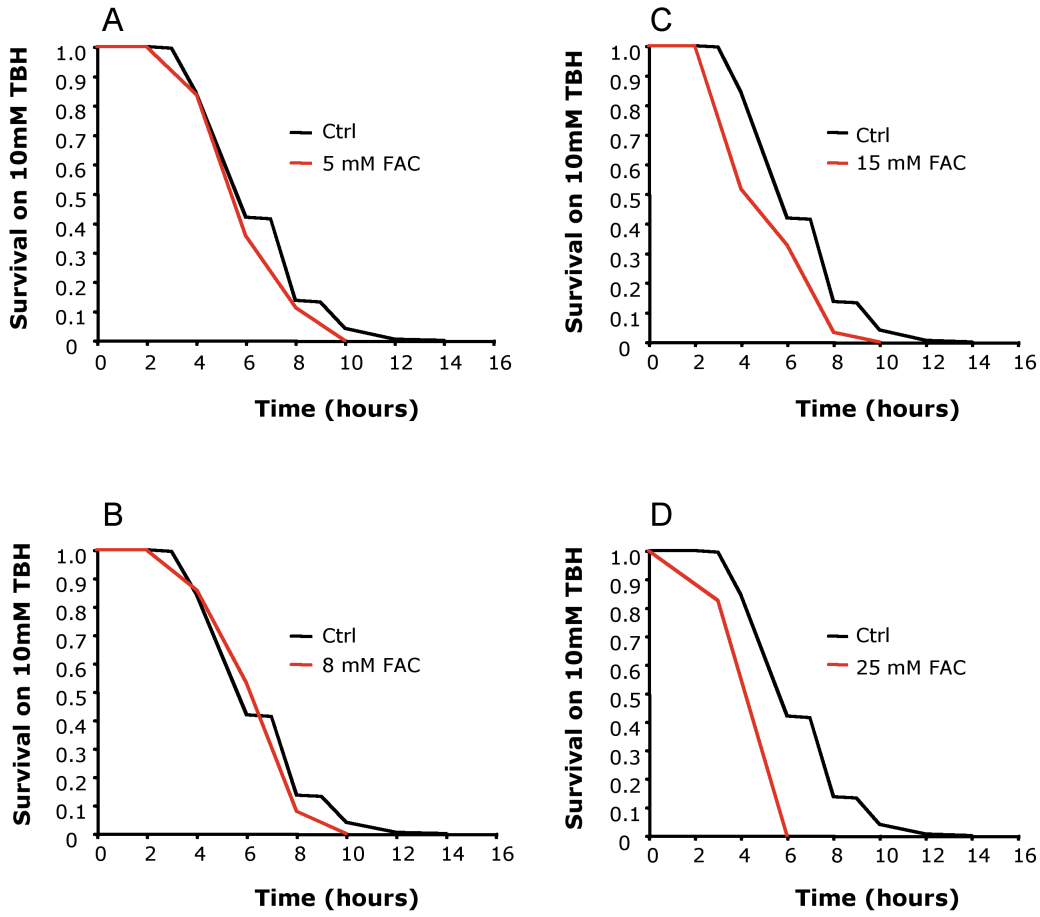


Figure 3.6: Iron supplementation increases peroxide toxicity in *C. elegans*. The trials were performed at room temperature on 10 mM TBH plates. Genotype: N2 wildtype. **(A-D)** Survival curves of wildtype worms pre-treated with 5 mM, 8 mM, 15 mM and 25 mM FAC compared to control animals without iron treatment. Statistical analysis is presented in Table 3.4.

Table 3.4: Iron supplementation increases peroxide toxicity in *C. elegans*. The trials were performed at room temperature on 10 mM TBH plates. Genotype: N2 wildtype, *p*, log rank test. [n] biological replicates number.

Strain	FAC (mM)	Deaths/ censored	Mean life span (hours)	% *vs.* ctrl	*p vs.* ctrl
Wildtype	Ctrl	**[C] 201/10**	**6.9**		
		[1] 25/0	6.2		
		[2] 29/0	5.6		
		[3] 43/4	7.7		
		[4] 58/2	6.8		
		[5] 46/4	7.4		
Wildtype	5	**[C] 62/4**	**6.6**	**-4.3**	**0.32**
		[1] 25/0	5.9	-4.8	0.27
		[3] 37/4	7	-9.1	0.21
Wildtype	8	**[C] 100/3**	**6.9**	**0**	**0.85**
		[4] 58/3	6.7	-1.5	0.88
		[5] 42/0	7.3	-1.5	0.35
Wildtype	15	**[C] 95/7**	**5.7**	**-17.4**	**<.0001**
		[2] 42/0	4.2	-25	<.0001
		[3] 53/7	6.9	-10.4	0.06
Wildtype	25	[1] 23/0	5.5	-11.3	0.06

Supplementation with 5 mM and 8 mM FAC did not cause a significant difference in survival upon 10 mM TBH compared to control animals (6.6 hours *vs.* 6.9 hours, p=0.32 and 6.9 hours *vs.* 6.9 hours, p=0.85, respectively). Increasing the FAC concentration to 15 mM caused a significant 16.4% reduction in survival compared to control animals (5.7 hours *vs.* 6.9 hours, p<.0001, respectively). In a single trial, worms exposed to 25 mM FAC also showed a 11.8% reduction in mean survival, although this difference did not quite reach statistical significance (5.5 hours *vs.* 6.2 hours, respectively, p=0.06).

These results show that increased iron levels can result in peroxide hypersensitivity in *C. elegans*. This effect was dose-dependent and results implied a dose-response threshold somewhere between 8 mM and 15 mM FAC. Since iron supplementation elevates oxidative damage levels and peroxide sensitivity, I next investigated the effect of iron treatment on *C. elegans* life span.

3.2.5. Iron supplementation reduces *C. elegans* life span

Does oxidative damage caused by free iron contribute to ageing in *C. elegans*? To investigate this, I tested the effect of different iron concentrations on life span. Wildtype worms were transferred as young adults to plates supplemented with different concentrations of FAC, ranging from 5-50 mM and their survival over the following weeks observed. The change of protocol to start trials with young adults instead of L4s was informed by prior tests with high iron concentrations (e.g. 400 mM and 600 mM), which had deleterious effects on *C. elegans* development (data not shown). To be consistent, I also

began life span measurements at lower iron concentrations using young adult. The trials were conducted at 20˚C without FUdR, except for trial [4] where 10 µM FUdR was used. To exclude offspring, the animals were transferred every day within the first week of the life span measurements. In case of FUdR usage, it was applied topically to the plates one day prior to the start of the experiment (see Materials & Methods, section 2.2.6.1.). The results are presented in Figure 3.7 and Table 3.5.

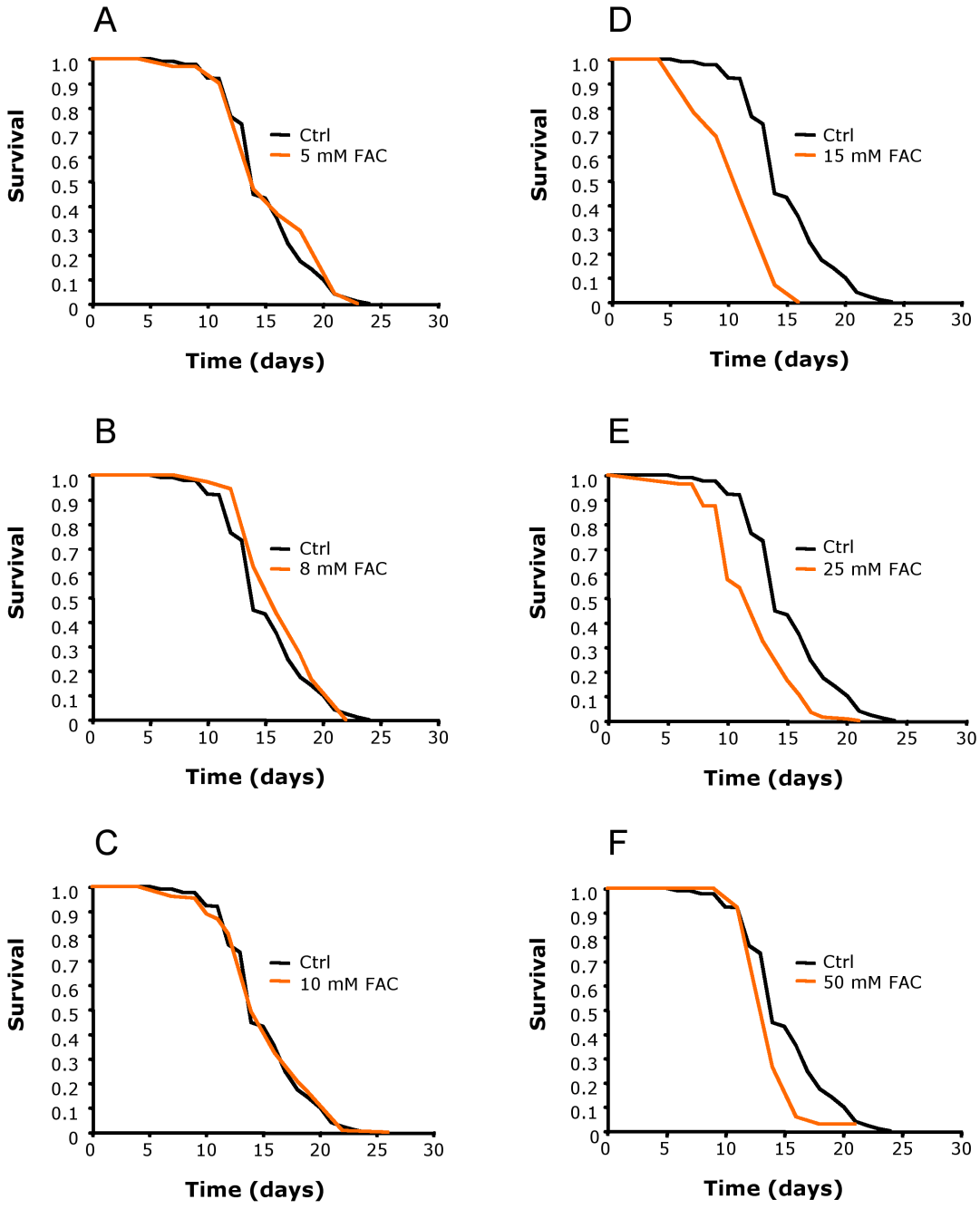


Figure 3.7: Effect of increased iron levels on *C. elegans* life span. The trials were performed at 20˚C without FUdR. Genotype: N2 wildtype. **(A-F)** Survival curves of worms on 5 mM, 8 mM, 10 mM, 15 mM, 25 mM and 50 mM FAC compared to control animals without iron treatment. Statistical analysis is presented in Table 3.5.

Table 3.5: Effect of increased iron levels on *C. elegans* life span. The trials were performed at 20˚C without FUdR, except of trial [4] 10 µM FUdR was used. Genotype: N2 wildtype, *p,* log rank test. [n] biological replicates number.

Strain	**FAC (mM)**	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* ctrl**	***p vs.* ctrl**
Wildtype	Ctrl	**[C] 214/132**	**15.9**		
		[1] 30/20	15.7		
		[2] 97/28	14.8		
		[3] 21/20	18.3		
		[4] 66/64	16.3		
Wildtype	5	[1] 28/22	16.2	+3.4	0.47
Wildtype	8	[4] 88/44	16.7	+2.5	0.42
Wildtype	10	**[C] 130/48**	**15.6**	**-1.9**	**0.98**
		[1] 30/20	13.2	-16	0.01
		[4] 100/28	16.3	-0	0.96
Wildtype	15	[1] 29/21	11.4	-27.4	<.0001
Wildtype	25	**[C] 153/12**	**12.2**	**-23.3**	**<.0001**
		[2] 123/2	11.5	-22.3	<.0001
		[3] 30/10	14.9	-18.6	<.0001
Wildtype	50	[3] 36/6	14.4	-21.3	<.0001

As for TBH sensitivity, life span was unaffected by lower iron concentrations, such as 5 mM (16.2 days *vs.* 15.9 days respectively, p=0.47), 8 mM (16.7 days *vs.* 15.9 days respectively, p=0.42) and 10 mM (15.6 days *vs.* 15.9 days respectively, p=0.98). However, in one of two trials 10 mM iron caused a decrease in life span (15.7 days *vs.* 13.2 days, p=0.01).

Worms showed significantly reduced mean life span upon exposure to higher iron concentrations, such as 15 mM (11.4 days *vs.* ctrl 15.9 days, p<.0001), 25 mM (12.2 days *vs.* ctrl 15.7 days, p<.0001) and 50 mM (14.4 days *vs.* ctrl 18.3 days, p<.0001). This indicates that with respect to life span the threshold for toxic iron concentrations in *C. elegans* lies between 10 mM and 15 mM. This is consistent with an earlier study, which reported 16.5 mM iron to be toxic to *C. elegans* (Gourley et al., 2003).

The control animals in life span trials [1], [2] and [4] showed mean life spans of 14.8-16.3 days. These values are unusually low relative to the expected 18-20 day life span range of N2 wildtype worms at 20˚C (Gems and Riddle, 2000). This would reflect a background or second site mutation, causing a reduced life span in the N2 cultivar used. The occurrence of mutations arising in a *C. elegans* culture is not that uncommon (Partridge and Gems, 2007). However, given that I tested the effect of different iron treatments on the same strain, the observed effects are informative.

Overall, these results show that exposure to 15 mM iron and higher concentrations are toxic to *C. elegans*. Tests to further investigate the role of iron in *C. elegans* ageing are presented in the next section.

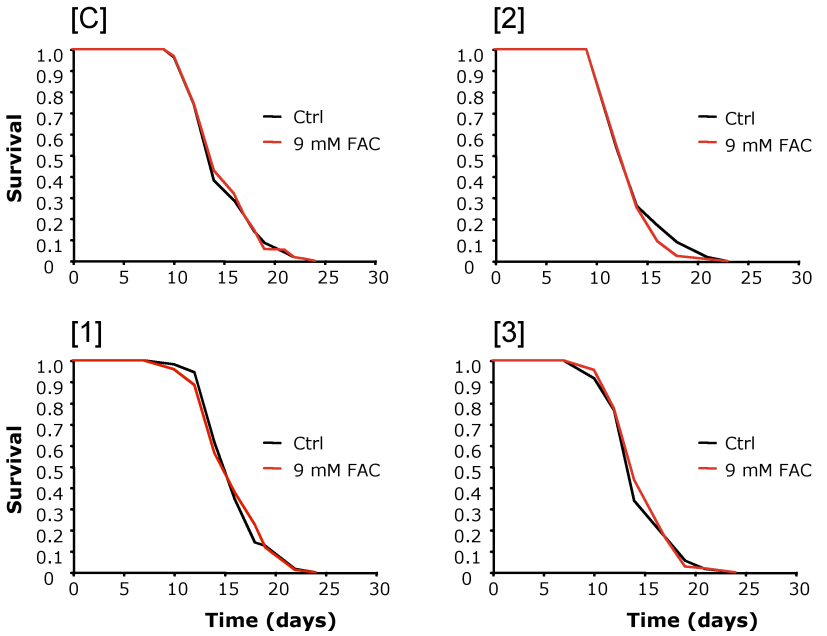
3.2.6. Effect of iron supplementation on ageing in *C. elegans*

In section 3.2.3.3., I showed that exposure to 15 mM iron significantly increased the carbonylation of proteins, a marker of oxidative damage, in *C. elegans*. Worms exposed to this iron concentration were also hypersensitive to peroxide toxicity (see section 3.2.4.) and short-lived (see section 3.2.5.).

This suggests that when iron levels are high, iron catalyzed molecular damage can shorten life span. The associated peroxide hypersensitivity is consistent with a role of this damage. What remains unclear is whether iron catalyzed molecular damage, particularly due to Fenton chemistry, plays any role in normal ageing. One way to address this is to ask: Is it possible to elevate iron levels to a degree that causes peroxide hypersensitivity (i.e. increases Fenton chemistry) but does not reduce life span? If yes, this would imply that Fenton chemistry do not limit normal life span. I therefore performed a series of trials to determine whether there exists a range of concentrations that cause peroxide hypersensitivity but do not shorten life span.

For life span measurements, young adult worms were transferred to control plates or plates supplemented with a final concentration of 9 mM FAC and scored over the next few weeks for their survival. In trial one, FUdR was applied topically to the plates one day before the start of the experiment in a final concentration of 10 µM. For TBH assays, young adult worms were transferred to control or 9 mM FAC plates, incubated for one day at 20˚C, and transferred to 10 mM TBH plates for oxidative stress measurements (see Materials & Methods, section 2.2.6.1. and 2.2.6.2.). The results are presented in Figure 3.8 and Table 3.6.

A) Combined life span data [C] and individual trials [1]-[3]



B) Combined TBH data [C] and individual trials [1]-[3]

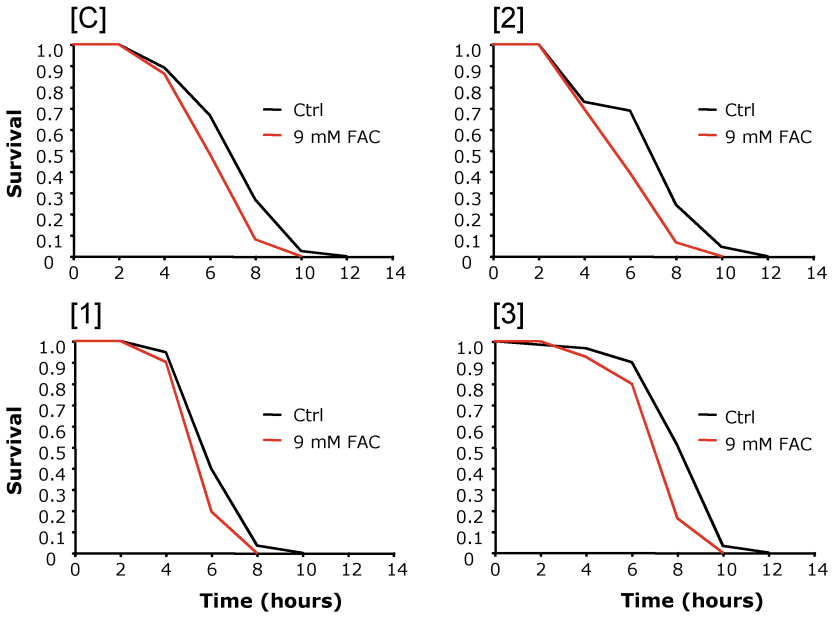


Figure 3.8: Effect of 9 mM FAC on *C. elegans* life span and peroxide toxicity. The life span trials were performed at 20˚C without FUdR and the TBH assays were performed at room temperature. Genotype: N2 wildtype. **(A)** Survival curves of wildtype worms on 9 mM FAC compared to control animals without FAC treatment. **[C]** Shows the life span curves of the combined data and **[1]**, **[2]** and **[3]** the individual trials. **(B)** Effect of 9 mM iron pre-treatment on survival on 10 mM TBH. Again, **[C]** represent the life span curves of the combined data and **[1]**, **[2]** and **[3]** the individual trials. Statistical analysis is presented in Table 3.6.

Table 3.6: Effect of 9 mM FAC on *C. elegans* life span and peroxide toxicity. The life span trials were performed at 20˚C, without FUdR, except of trial [1] using 10 µM FUdR, whereas TBH assays were performed at room temperature. Genotype: N2 wildtype, *p,* log rank test. [n] biological replicates number.

Strain	**FAC (mM)**	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* ctrl**	***p vs.* ctrl**
Wildtype	Ctrl	**[C] 306/122**	**15.1**		
		[1] 66/64	16.3		
		[2] 115/21	14.2		
		[3] 125/37	14.9		
Wildtype	9	**[C] 287/103**	**15.1**	**0**	**0.77**
		[1] 95/40	16.2	-0.6	0.81
		[2] 83/37	13.9	-2.1	0.43
		[3] 109/30	15.2	+2	0.65
	+ 10 mM TBH				
Wildtype	Ctrl	**[C] 165/6**	**7.7**		
		[1] 58/2	6.8		
		[2] 46/4	7.4		
		[3] 61/0	8.8		
Wildtype	9	**[C] 138/19**	**6.9**	**-10.4**	**<.0001**
		[1] 51/9	6.2	-8.8	0.01
		[2] 32/9	6.3	-14.9	0.007
		[3] 55/1	7.8	-11.4	0.0001

In three trials the life span measurements showed no significant difference between control worms and worms exposed to 9 mM iron (range: 14.2-16.3 days *vs.* 13.9-16.2 days respectively, p=0.77). Therefore this iron concentration is not sufficient to shorten life span. On the other hand, the pre-treatment with 9 mM iron significantly decreased mean survival by 10.4% (8.8%-14.9%) upon TBH compared to control animals (range: 6.2-7.8 hours *vs.* 6.8-8.8 hours, respectively, p<.0001).

These results showed that 9 mM FAC increases sensitivity to peroxides toxicity without reducing life span. This suggests that 9 mM FAC increases Fenton chemistry without shortening life span. This in turn suggests that the levels of Fenton chemistry in *C. elegans* under standard culture conditions are not limiting for life span, i.e. that Fenton chemistry contributes little to normal ageing in *C. elegans*.

3.2.7. Effect of an iron chelator on free iron levels in *C. elegans*

If *C. elegans* is limited by iron catalyzed oxidative damage, then lowering levels of free iron should increase life span. I therefore investigated the effect of lowered free iron levels on *C. elegans* using the iron chelator deferoxamine (DF). DF is reported to decrease iron levels in mammals (Eisenstein, 2000; Hentze and Kuhn, 1996; Theil and Eisenstein, 2000) as well as in *C. elegans* (Gourley et al., 2003).

Every cell in biological systems has a labile iron pool available for metabolic processes. By chelating free iron available to the worms, the iron that catalyzes production of the hydroxyl radical via the Fenton Reaction is

limited. We chose the iron chelator DF at a concentration of 100 μM since it was used successfully in an earlier study (Gourley et al., 2003).

3.2.7.1. Effect of deferoxamine on free iron levels in *C. elegans*

To measure the effect of deferoxamine on free iron levels within the worms, Mustafa Alam performed EPR measurements under my supervision. Stage synchronized worms were transferred as young adults to NGM plates containing 100 μM DF, incubated at 20˚C overnight and then collected for EPR measurements (see Materials & Methods, section 2.2.14.). The results are presented in Figure 3.9 and Table 3.7.

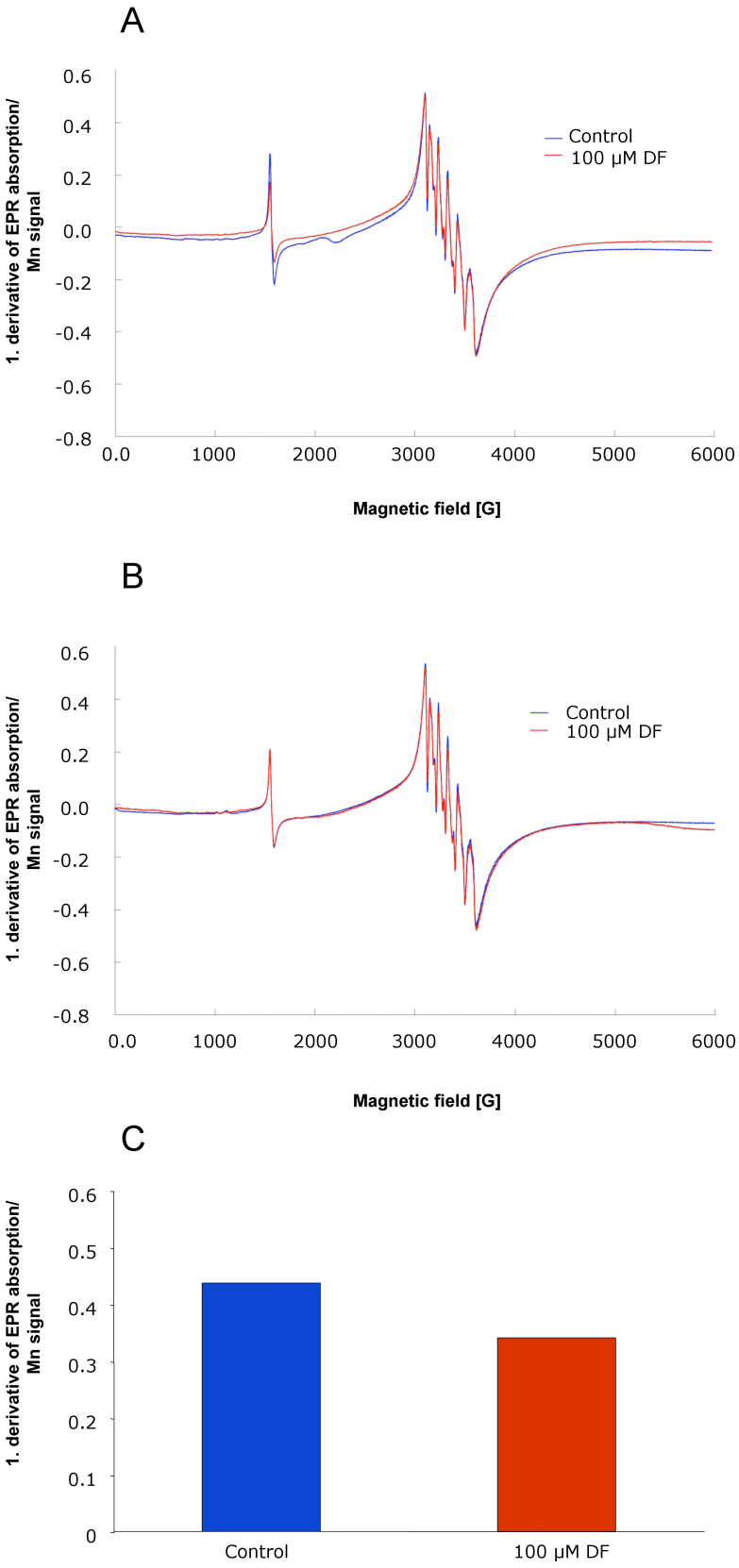


Figure 3.9: Effect of deferoxamine on free iron levels in the worms. (A-B) The two individual EPR spectra of N2 wildtype worms with and without DF treatment. **(C)** The columns show the mean of two biological replicates. Since we had only two biological replicates no statistical analysis could be conducted.

Table 3.7: Effect of deferoxamine on free iron levels in the worms. Genotype: N2 wildtype. The numbers for each EPR measurement represent the length of the iron peak. Since the measurements were taken only twice, no statistical analysis could be conducted.

Strain	DF (μM)	Size of iron peak (arbitrary units)	% *vs.* ctrl
Wildtype	Ctrl	**[C] 0.43** [1] 0.50 [2] 0.37	
Wildtype	100	**[C] 0.34** [1] 0.30 [2] 0.37	**-20.9** -40 -0

The mean length of the peak corresponding to the free iron levels was 20.9% shorter in worms treated with DF compared to control animals. Measurement [1] showed a 40% shorter peak, indicating reduced free iron levels upon DF treatment, whereas measurement [2] showed no difference in free iron levels to control animals. Since each measurement represents one biological replicate, it was not possible to determine whether the reduction in free iron is statistically significant. These results suggest that DF probably does lower free iron levels in *C. elegans*, though further trials would be necessary to establish this with certainty.

3.2.7.2. Effect of deferoxamine on *Pftn-1::GFP* expression

As a further test of effects of DF on free iron levels, I tested whether it decreases *Pftn-1::GFP* expression. I again used the GFP reporter strain GA631 *lin-15(n765ts) X wuIs177 [Pftn-1::GFP, lin-15(+)]*. I previously showed that *Pftn-1::GFP* expression can be used as a sensor of intracellular iron levels. Worms were raised on 100 μM DF plates and GFP levels were measured on the fifth day after egg lay using the plate-reader (see Materials & Methods, section 2.2.5.).

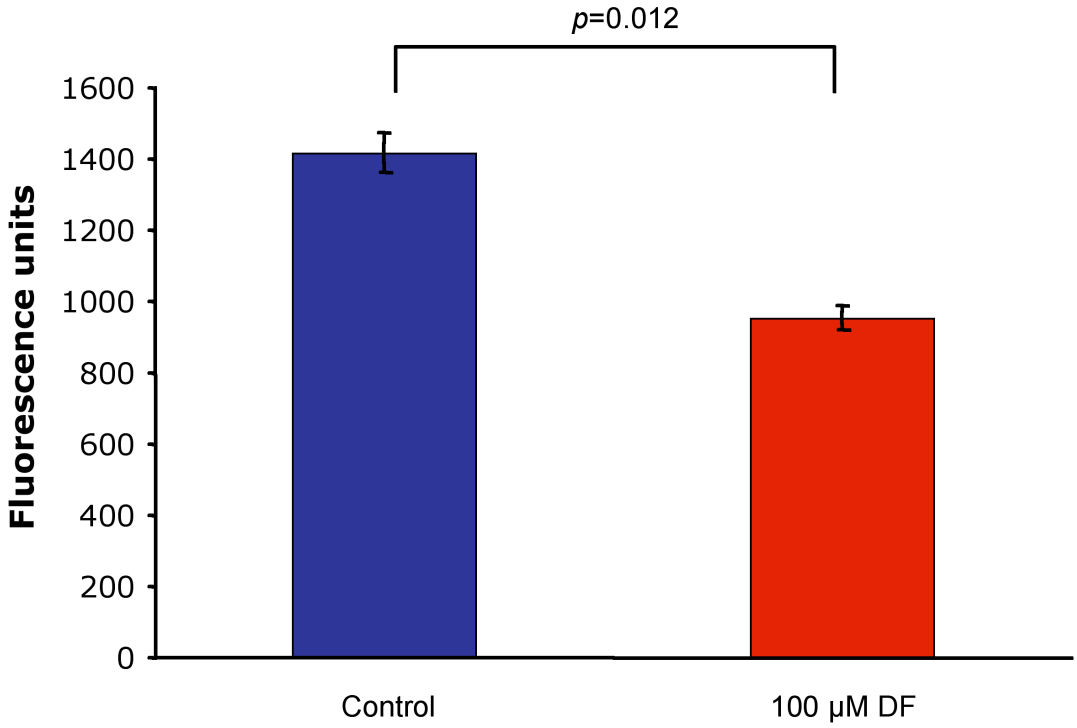


Figure 3.10: Expression of *Pftn-1::GFP* on 100 µM deferoxamine. GFP expression on 100 µM DF was compared to control animals. The columns represent the mean of three biological replicates, the error bars show the S.E.M. and statistical analysis was performed with the Student's T-test.

The GFP fluorescence intensity in the reporter strain showed a 32.7% decrease in animals treated with 100 μM DF (p=0.012). This implies that treatment with this iron chelator reduces iron levels within the worms, supporting the findings of the EPR tests in the previous section.

3.2.8. Effect of deferoxamine on peroxide resistance

If reduction of DF reduced the occurrence of Fenton chemistry, then it should increase resistance to peroxide toxicity. To test this, I performed TBH resistance assays. For this, young adult N2 wildtype worms were transferred to control and 100 μM DF plates, incubated for one day at 20˚C, and transferred to 10 mM TBH plates and scored every two hours for survival (see Materials & Methods, section 2.2.6.2.). The results are presented in Figure 3.11 and Table 3.8.

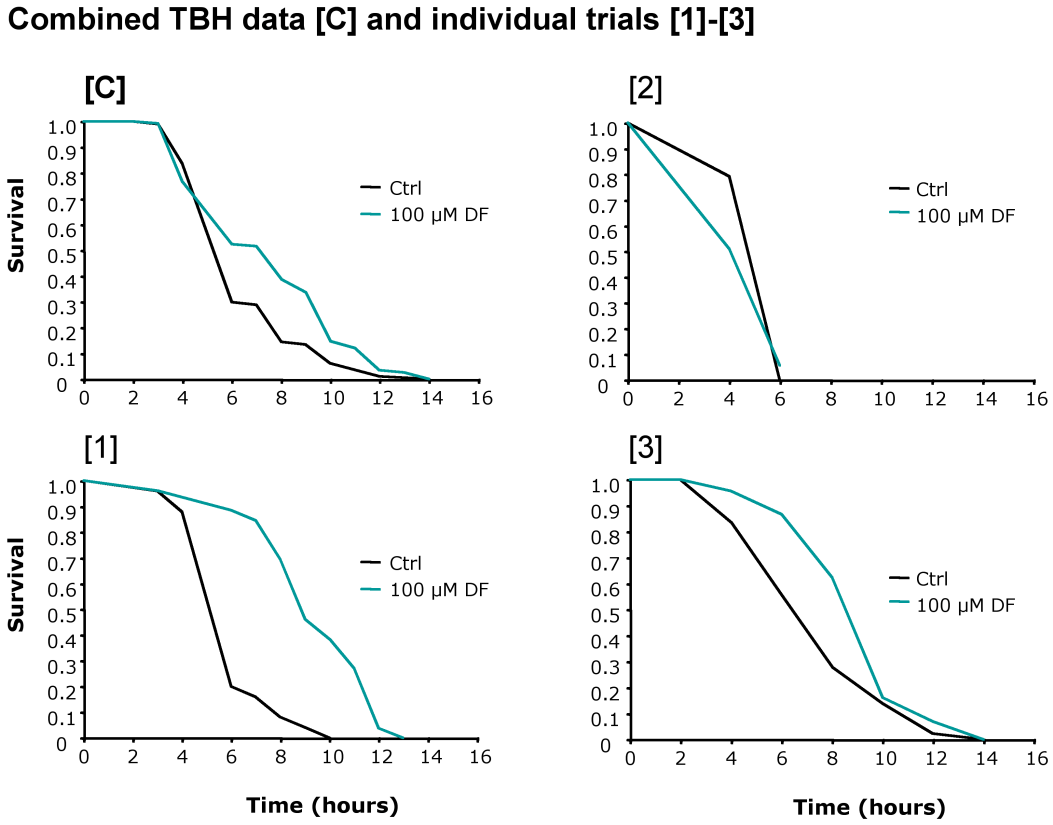


Figure 3.11: Effect of deferoxamine on peroxide toxicity in *C. elegans*. The TBH trials were performed at room temperature. Genotypes: N2 wildtype. Effect of 100 µM DF treatment on survival on 10 mM TBH. Again**, [C]** shows the combined data of the survival on TBH and **[1]**, **[2]** and **[3]** represent the individual trials. Statistical analysis is presented in Table 3.8.

Table 3.8: Effect of deferoxamine on peroxide toxicity in *C. elegans*. Genotypes: N2 wildtype, *p,* log rank test. [n] biological replicates number.

Strain	Treatment	Deaths/ censored	Mean life span (days)	% *vs.* ctrl	*p vs.* ctrl
Wildtype	Ctrl	**[C] 97/4**	**6.7**		
		[1] 25/0	6.2		
		[2] 29/0	5.6		
		[3] 43/4	7.7		
Wildtype	100 µM DF	**[C] 120/8**	**7.6**	**+13.4**	**0.008**
		[1] 26/0	9.5	+53.2	<.0001
		[2] 50/3	5	-10.7	0.08
		[3] 44/5	9.3	+20.7	0.005

The summed data showed an overall increased resistance to TBH after 100 μM DF exposure (6.7 hours *vs.* 7.6 hours, p=0.008). In trials [1] and [3], the DF treated worms showed significant 53.2% and 20.7% increase in survival upon 10 mM TBH treatment (6.2 hours *vs.* 9.5 hours, respectively, p<.0001 and 7.7 hours *vs.* 9.3 hours, respectively, p=0.005). However, the DF treated worms in trial [2] showed a 10.7% lower mean survival upon treatment with TBH (5.6 hours *vs.* 5 hours, respectively), but this difference was not significant (p=0.08). Therefore it seems that overall 100 μM DF increases resistance to 10 mM TBH. This suggests that by lowering iron levels, DF reduces Fenton chemistry in *C. elegans*. The next question is: Does this have any effect on the health of the worm?

3.2.8.1. Effect of deferoxamine on oxidative damage in *C. elegans*

Increased exposure to iron can increase oxidative damage levels, but does reduction of iron levels by chelation reduce it? To test this I used oxyblots to measure the effect of DF on the amount of carbonylated proteins.

Synchronized worms were transferred as young adults to control plates or plates supplemented with DF to a final concentration of 100 μM. These were incubated overnight and then collected the next day for carbonylated protein measurement (see Materials & Methods, section 2.2.13.). The result is presented in Figure 3.12.

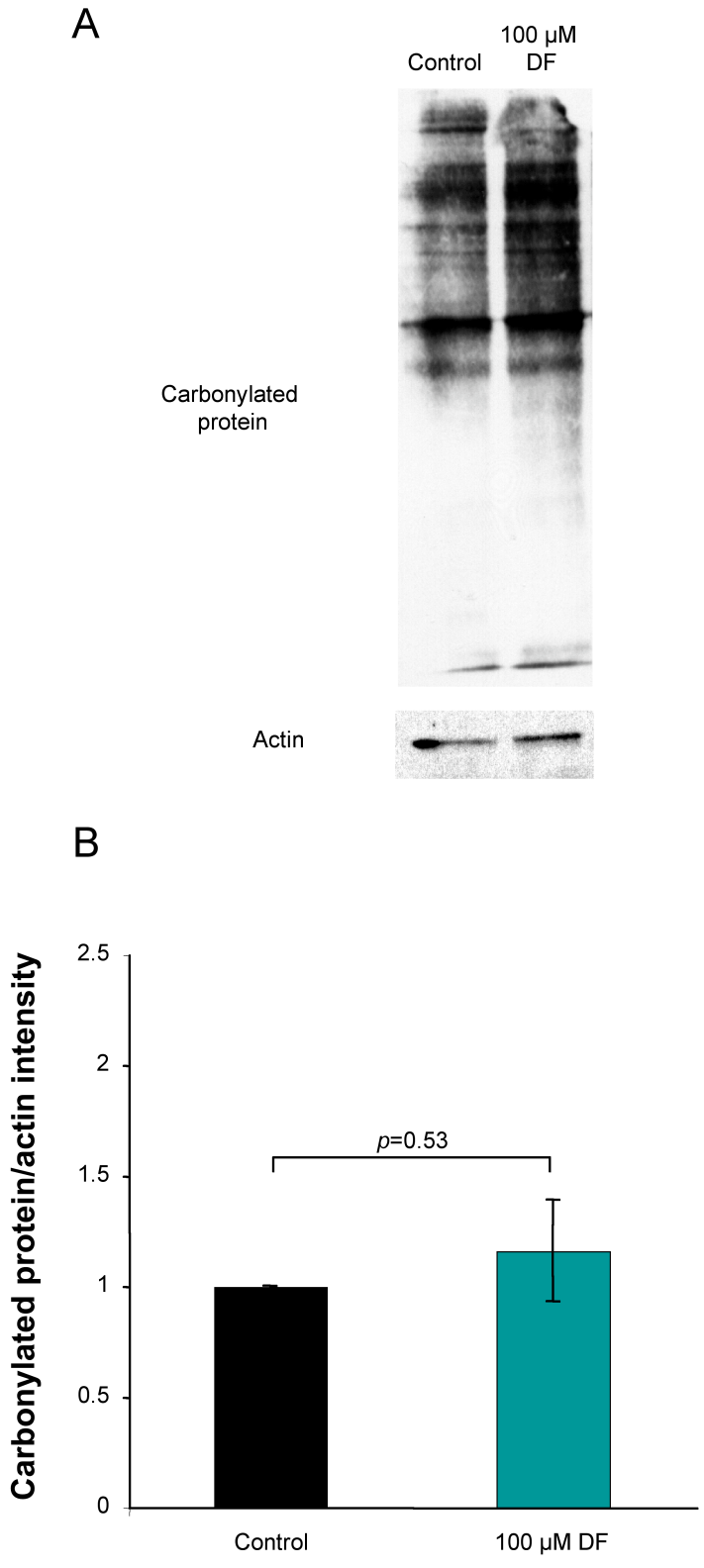


Figure 3.12: Effect of deferoxamine on protein oxidation levels in *C. elegans*. (A) A representative Oxyblot showing the carbonylated protein and actin levels**. (B)** Statistical analysis of four biological replicates, two performed by Dr Filipe Cabreiro, two by myself showing carbonylated protein against actin intensity. The error bars show the S.E.M. and statistical analysis was performed with the Student's T-test.

Treatment with 100 μM DF did not change the level of protein carbonylation in worms. Worms treated with the iron chelator showed a 16% higher protein carbonylation, but this difference was not significant (p=0.53). This could imply that under standard culture conditions, free iron contributes little to protein oxidation, at least in young adults. The effect of DF in *C. elegans* ageing is investigated in the next section.

3.2.9. Effect of deferoxamine on *C. elegans* ageing

To determine the effect of 100 μM DF on ageing in *C. elegans*, I performed life span measurements. I used the strain SS104 *(glp-4(bn2) I*) for the life span measurements. The gene *glp-4* is necessary for normal germ cell development and proliferation. Temperature sensitive mutations in *glp-4* such as *bn2* result in *C. elegans* infertility at higher temperatures, which is convenient for life span measurements. Worms were maintained on control plates and plates supplemented with a final concentration of 100 μM DF. They were raised at 15°C, then shifted to 25°C at L4 stage, and then monitored over the next few weeks for their survival. Results are shown in Figure 3.13 and Table 3.9.

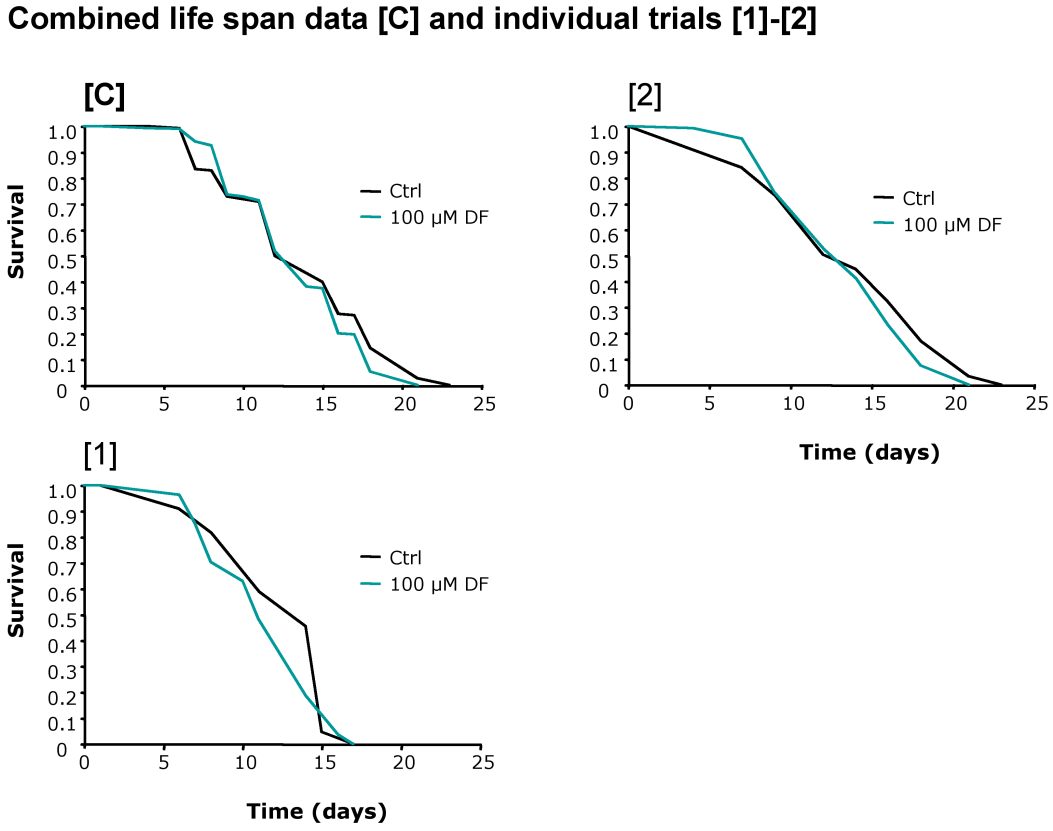


Figure 3.13: Effect of deferoxamine on life span in *C. elegans*. The life span trials were performed at 25˚C. Genotypes: SS104 *glp-4(bn2) I.* Survival curves of wildtype worms with 100 µM DF treatment compared to control animals. **[C]** Shows the life span curve of the combined data and **[1]** and **[2]** represent the corresponding individual trials. Statistical analysis is presented in Table 3.9.

Table 3.9: Effect of deferoxamine on life span in *C. elegans*. The life span trials were performed at 25˚C. Genotypes: SS104 *glp-4(bn2) I*, *p,* log rank test. [n] biological replicates number.

Strain	Treatment	Deaths/ censored	Mean life span (days)	% *vs.* ctrl	*p vs.* ctrl
SS104	Ctrl	**[C] 133/22**	**13.9**		
		[1] 22/8	12.5		
		[2] 111/14	14.2		
SS104	100 μM DF	**[C] 144/11**	**13.3**	**-4.3**	**0.07**
		[1] 27/3	11.6	-7.2	0.23
		[2] 117/8	13.7	-3.5	0.09

Life span data from two independent trials showed no significant difference between control animals and worms treated with 100 μM DF (12.5 days *vs.* 11.6 days, p=0.23 and 14.2 days *vs.* 13.7 days, p=0.09). Thus, reducing iron levels by treatment with 100 μM DF has no effect on *C. elegans* life span.

In conclusion, 100 μM DF reduces iron levels *in vivo* and this increases peroxide resistance consistent with reduced Fenton chemistry. However, this neither reduces oxidative damage nor increases life span. These results suggest that free iron levels, and the Fenton reaction contribute little to ageing in *C. elegans*. This may be because Fenton chemistry does not contribute significantly to molecular damage under standard conditions. Alternatively, it could be because molecular damage contributes little to *C. elegans* ageing.

3.3. Discussion

In this chapter, I first tested whether up-regulation of *ftn-1* might contribute to *daf-2* mutant longevity. I then asked whether free iron levels have any influence at all on *C. elegans* ageing.

3.3.1. *ftn-1* is not important in *daf-2* mutant longevity

Given the role of iron in oxidative stress, and its postulated role in ageing, one might expect it to be beneficial to have elevated levels of iron storage proteins, such as FTN-1. The data on *daf-2* mutants presented in this chapter did not reveal any such benefit. Knockdown of *ftn-1* expression had no affect on life span in either *daf-2* mutants or control animals. This implies that *ftn-1* is not important in *C. elegans* longevity assurance. The possibility that up-regulation of *ftn-2*, the other homologue of mammalian heavy chain ferritin in *C. elegans*, compensates for the loss of *ftn-1* is one potential explanation. However, simultaneous RNAi of both *ftn-1* and *ftn-2* had no effect on life span either, excluding this possibility.

One interpretation of these results is that metal homeostasis more broadly is not important for longevity assurance. This interpretation is supported by a study that showed that another class of proteins contributing to heavy metal homeostasis and detoxification, metallothioneins, is not essential for normal *C. elegans* wildtype life span (Hughes and Sturzenbaum, 2007). However, reduced expression levels of *mtl-1* (which encodes a metallothionein) was reported to slightly decrease *daf-2* mutant longevity (Murphy et al., 2003) and metallothioneins have been shown to have

protective effects to metal stress and metal catalyzed oxidative stress (Barsyte et al., 2001; Lazo et al., 1995; Michalska and Choo, 1993). Thus it remains possible that metallothioneins contribute to longevity by protecting against a broader range of ROS, such as superoxide, hydroxyl radical, nitric oxide and peroxinitrite, by binding various metals (Nielsen et al., 2007), whereas ferritins are specialised on binding iron and therefore only influence the ROS involved in the Fenton chemistry.

ftn-1 is not the first example of an antioxidant defence gene found to be non-essential for normal life span. For example deletion of Mn SOD genes *sod-2* and *sod-3* did not reduce life span in either wildtype or *daf-2* mutant background (Chavez et al., 2007; Doonan et al., 2008; Honda et al., 2008; Yang et al., 2007; Yen et al., 2009). Some studies even showed an increase in life span in *sod-2* or *sod-3* mutants (Honda et al., 2010; Van Raamsdonk and Hekimi, 2009), but this was not seen in other studies (Doonan et al., 2008; Yang et al., 2007; Yen et al., 2009). Furthermore, loss of *ctl-1* has no effect on *C. elegans* life span (Petriv and Rachubinski, 2004) see Introduction, section 1.5.).

3.3.2. *ftn-1* contributes weakly to resistance to peroxide stress

Results presented confirmed that *daf-2* mutants are resistant to oxidative stress induced via TBH, as they also are to various other forms of oxidative stress agents (Honda and Honda, 1999; Larsen, 1993; Vanfleteren, 1993). Knockdown of *ftn-1* expression significantly decreased resistance to oxidative stress in wildtype worms and probably also in *daf-2* mutants. Thus, *ftn-1* contributes to resistance to oxidative stress in *C. elegans*. This supports

the view that ferritin limits hydroxyl production by reducing levels of free iron that would otherwise feed the Fenton reaction.

ftn-1 RNAi caused similar small reductions in both *daf-2(+)* and *daf-2* mutant worms (though in the latter case this did not quite reach significance, p=0.06). This suggests that the increase in expression of *ftn-1* is not a major contributor to the increased oxidative stress resistance of *daf-2* mutants. The lack of effect of *ftn-1* on life span could reflect either its minor role in controlling Fenton reaction levels, or the unimportance of the Fenton reaction in ageing. To determine this, I tested the effect of manipulating iron levels on *C. elegans*.

3.3.3. Effects of iron supplementation on *C. elegans*

I first established protocols for verifiable increasing free iron levels *in vivo* in *C. elegans*. EPR and *Pftn-1::GFP* measurements, confirmed that iron supplementation increased free iron levels within the worms in a dose-dependent matter. Increased free iron, as expected increased peroxide sensitivity and oxidative damage measured as increased protein carbonylation. Iron supplementation up to 10 mM or higher decreased life span. A plausible interpretation of these results is that elevated free iron increases the Fenton reaction, thereby increase oxidative damage and decrease life span. Other modes of iron mediated oxidative damage are also likely to play a role.

However, the initial question here is not whether iron can shorten life span, but whether iron levels and particularly Fenton chemistry limit life span under standard culture conditions. In other words: Does iron-generated

oxidative damage contribute to ageing? To probe this I asked: Are there concentrations of iron that increase peroxide sensitivity, without reducing life span? My results support the view that iron can induce oxidative stress and damage in *C. elegans*.

Before performing this experiment, we foresaw two likely outcomes. Firstly, the iron dose thresholds for peroxide toxicity and life span might be identical, consistent with a role of Fenton chemistry in ageing. Secondly, the threshold for peroxide toxicity might be marked by lower than that for life span, which would suggests that Fenton chemistry does not limit life span. The actual result was somewhere in between, insofar as the peroxide toxicity threshold was lower, but not markedly. This result is ambiguous. It could imply that with 9 mM FAC supplementation Fenton chemistry is raised, but does not shorten life span. Alternatively, it could be that Fenton chemistry is only elevated at 9 mM FAC when peroxide levels are elevated by TBH supplementation. A more informative approach is to reduce free iron levels and see whether life span is increased.

3.3.4. Effect of reduced iron levels in *C. elegans*

Overall, the measured effects of 100 µM DF on *C. elegans* imply that this iron chelator effectively lowers free iron levels *in vivo*. EPR data shows a reduction in free iron levels in one of two trials. *Pftn-1::GFP* levels are significantly reduced. Resistance to peroxide (TBH) is increased. But this neither reduces protein oxidation nor increases life span. The lack of effect on protein oxidation suggests that protein oxidation levels in young adult worms are not significantly affected by free iron under standard culture conditions.

However, it is conceivable that greater reduction in free iron levels would lower protein carbonyl levels. It is also possible that other forms of molecular damage are reduced, e.g. damage to nuclear or mitochondrial DNA, or to lipids. It is also possible that DF treatment might slow the age increase in protein oxidation. However, even if lowering free iron levels with 100 μM DF does reduce damage levels, this has no impact on ageing.

The results presented here suggest that under standard culture conditions, levels of free iron are not limiting for ageing. This suggests that OH• production by the Fenton reaction is not important in *C. elegans* ageing. To test this further, I attempted to more robustly reduce free iron levels in *C. elegans* by means of over-expression of *ftn-1*. The results are described in the next chapter.

Chapter 4: Effect of over-expressing *ftn-1* on oxidative damage and ageing in *C. elegans*

4.1. Introduction

4.1.1. Generation of *ftn-1* over-expressing lines

In the previous chapter I tested the role of free iron oxidative damage and ageing in *C. elegans*. To do so, I manipulated iron levels by iron supplementation or chelation. The results implied that given standard conditions, free iron contributes little to oxidative damage or ageing in *C. elegans*.

I next tried a different approach to test the role of free iron in ageing by generating transgenic *ftn-1* over-expressing lines (see Materials & Methods, section 2.2.10.). To obtain transgenic animals containing multiple copies of *ftn-1* I used the microinjection technique.

Romney at al. identified the core regulatory region of *ftn-1* as a 63 bp sequence 693 bp upstream of the translational start site (Romney et al., 2008). I amplified the complete *ftn-1* coding region including 3860 bp upstream and 1048 bp downstream of the coding region using *C. elegans* genomic DNA as template. The 3860 bp *ftn-1* region included the core promoter, and the downstream region the 3'UTR. I co-injected the amplified DNA together with a GFP marker for selection of transgenic animals (*coel::GFP*) into the distal gonad syncytium of N2 wildtype worms. Transgenic animals produced by injection typically carry large extrachromosomal arrays that contain many copies of the co-injected DNAs. These repetitive arrays are

usually unstable to cell division but can become inheritable, leding to genetically mosaic transformant animals.

4.1.2. The *coel::GFP* marker

Since the transparent nature of *C. elegans* allows the usage of fluorescent proteins to study gene expression of various genes and transgenes, I used a green fluorescent protein (GFP) as a scoreable phenotypic marker, *coel::GFP*. This contains the *unc-122* promoter fused to GFP within the pPD95.77 backbone. The *unc-122* gene is expressed in coelomocytes, which are large cells full of vesicles, situated in the *C. elegans* pseudocoelomic space. Adult hermaphrodites have six of these cells while adult males have five. The *unc-122* promoter drives expression of GFP in all of them.

The aim of this chapter was to efficiently lower free iron levels *in vivo* by over-expression of *ftn-1*. It could then be asked whether this increases oxidative stress resistance, reduces oxidative damage levels and increases life span.

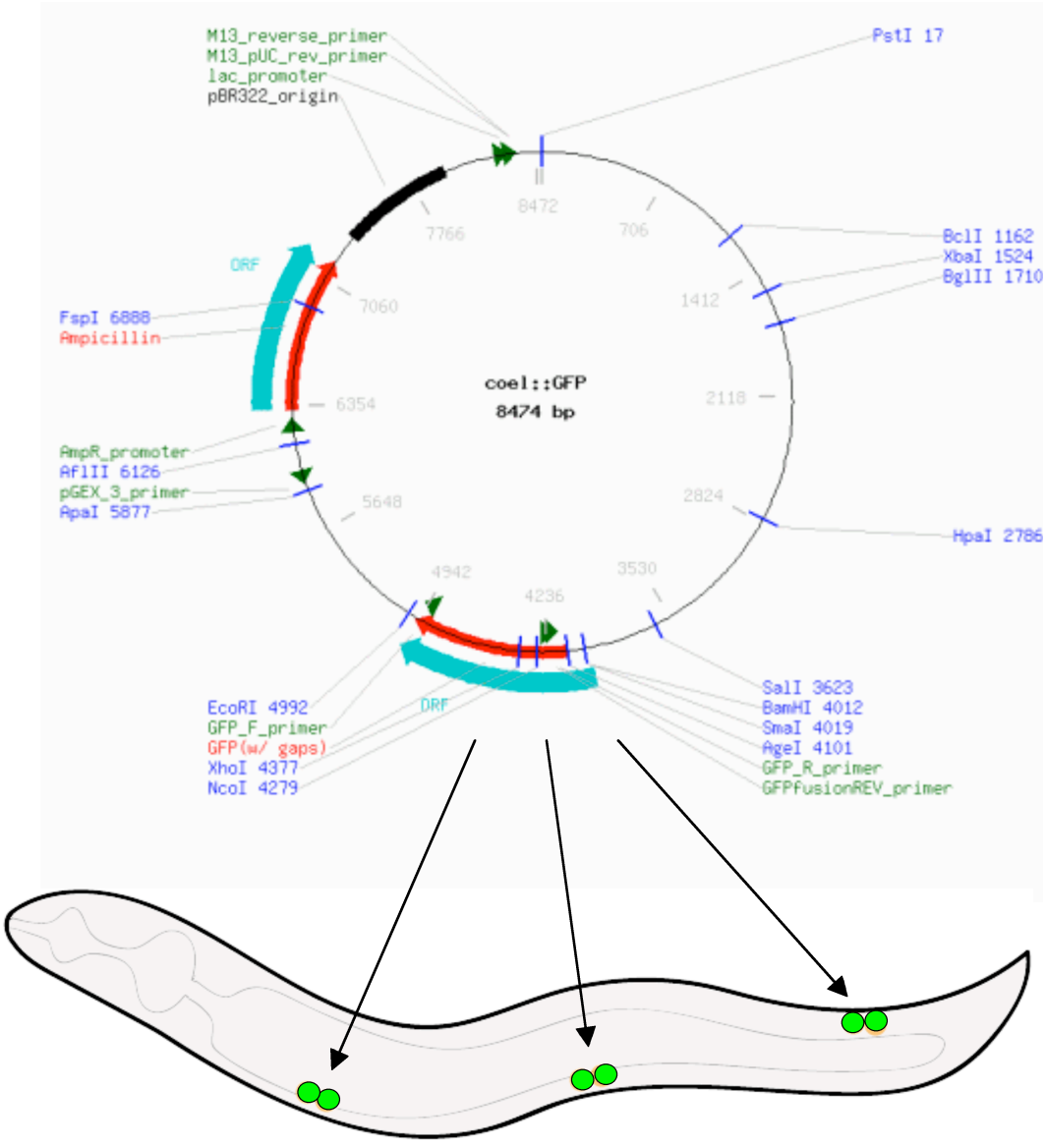


Figure 4.1: Expression pattern of the *coel::GFP* plasmid in *C. elegans*. The *unc-122* promoter induces GFP expression in the six coelomocytes, as indicated in the Figure. Sources: The plasmid map from Addgene website, and the *unc-122* expression pattern from WormBase.

4.2. Results

4.2.1. Selection of *ftn-1* over-expressing (OE) lines

Three stable transgenic *ftn-1* over-expressing lines were generated with high transmission rates, GA902 (*wuEx185 [Pftn-1::ftn-1::ftn-1 3'UTR + coel::GFP]*), GA903 (*wuEx186 [Pftn-1::ftn-1::ftn-1 3'UTR + coel::GFP]*) and GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-1 3'UTR + coel::GFP]*) by Dr David Weinkove. The transmission rate is the proportion of offspring that inherit the transgene array. The *wuEx185* transgene array showed a transmission rate of 70%, *wuEx186* one of 68% and *wu187* 65%.

As genetic control I myself injected wildtype worms only with the GFP marker, excluding the *ftn-1* fragment. I obtained one stable line, GA901 (*wuEx188 [coel::GFP]*). This strain allowed me to control for effects of the *coel::GFP* marker on *C. elegans*. The transmission rate of this transgenic line was 50%. To determine whether each of the three transgenic lines injected with the extra copies of *ftn-1* and the *coel::GFP* plasmid, over-expressed *ftn-1* and at what levels, I performed RT-PCR (see Materials & Methods, section 2.2.12.). I added wildtype and the injection marker control *wuEx188* as controls. The results are presented in Figure 4.2.

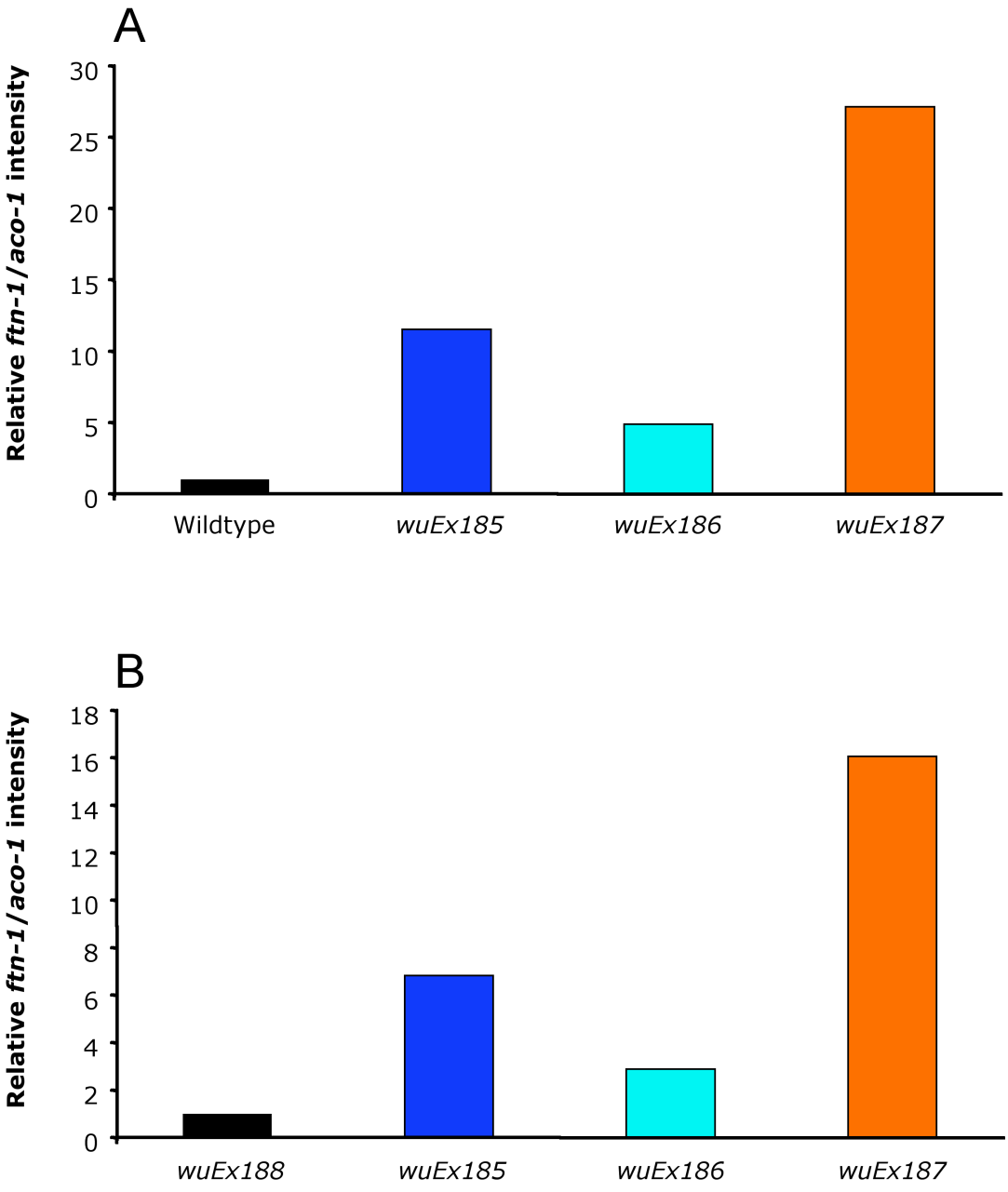


Figure 4.2: *ftn-1* mRNA levels in the four transgenic lines. Genotypes: GA902 (*wuEx185 [Pftn-1::ftn-1::ftn-1 3'UTR + coel::GFP]*), GA903 (*wuEx186 [Pftn-1::ftn-1::ftn-1 3'UTR + coel::GFP]*), GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-1 3'UTR + coel::GFP]*), GA901 (*wuEx188 [coel::GFP]*). **(A)** *ftn-1* mRNA compared against *aco-1* levels standardized to wildtype animals in the three transgenic strains. **(B)** Shows the *ftn-1* mRNA against *aco-1* levels of the three transgenic lines standardized to the injection marker control. Each column represents the mean of three technical replicates. Since this RT-PCR was conducted on a single biological replicate, no statistical analysis could be conducted.

RT-PCR tests implied that *ftn-1* mRNA levels are higher in all three lines relative to both wildtype and *coel::GFP* control (*wuEx185* 11.5% and 6.8%, respectively, *wuEx186* 4.9% and 2.9%, respectively, *wuEx187* 27.1% and 16.1%, respectively). These are likely to be underestimates given that there is only partial transmission of the transgene array, and the character of some transformants are mosaic. It is impossible to determine if and how significant the increase in *ftn-1* mRNA levels is in the individual lines without further biological replicates.

GA904, the line with the *wuEx187* transgene, had the lowest transmission rate of the three lines, but still showed the highest *ftn-1* over-expression implying a particularly high level of *ftn-1* over-expression. I therefore choose this line for all further experiments presented in this chapter. For GA904, I repeated the RT-PCR twice more to confirm the elevated *ftn-1* mRNA levels shown in the preliminary RT-PCR. The results are presented in Figure 4.3.

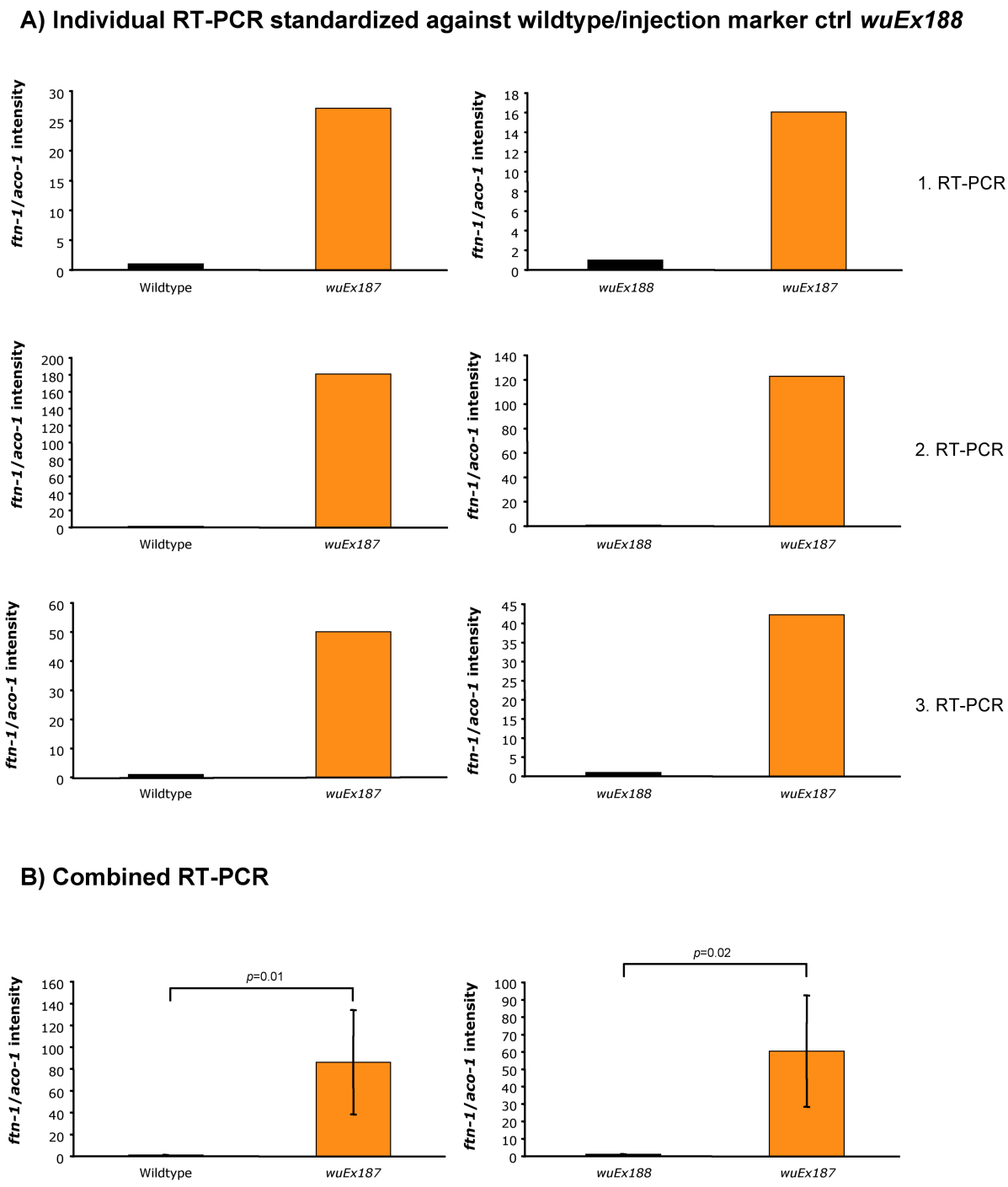


Figure 4.3: *ftn-1* mRNA levels of the transgenic line GA904 (*wuEx187*) standardized to wildtype and injection marker control GA901 (*wuEx188*). (A) Individual RT-PCRs showing the *ftn-1* mRNA levels of the transgenic *wuEx187* line standardized against N2 wildtype and *wuEx188*. **(B)** The combined data of the individual RT-PCRs and the statistical analysis. Each column shows the mean of the individual RT-PCR. The error bars represent the standard error of the mean (S.E.M) and statistical analysis was conducted with the Student's T-test.

The individual RT-PCR tests showed elevated *ftn-1* mRNA levels in the transgenic line GA904 (*wuEx187*) compared to wildtype and injection marker control animals (27.1, 180.9 and 50.1-fold increase *vs.* wildtype; 16.1, 122.7 and 42.2-fold increase *vs. wuEx188,* Figure 4.3 A). The combined data shows a 86-fold increase of *ftn-1* mRNA levels to wildtype animals (p=0.01, Figure 4.3 B) and a 60.3-fold increase to the injection marker control strain (p=0.02, Figure 4.3 B).

The variation between the individual RT-PCRs may be caused by variation in the transmission rate or the degree of transformant mosaicism. It is therefore likely that the over-expression level is underestimated to varying degrees. By this view, the data indicates that GA904 transformants have at least 180-fold higher *ftn-1* mRNA levels compared to wildtype and 122-fold higher *ftn-1* mRNA levels compared to the transgenic control animals. Since *ftn-1* is post-transcriptionally regulated, I tried to verify elevated FTN-1 levels for this line using western blots. However, all attempts to find an antibody binding to *C. elegans* FTN-1 failed. For reasons of simplicity, the GA904 (*wuEx187*) line will be described as the *ftn-1* over-expresser (OE), and the GA901 (*wuEx188*) as *ftn-1* OE control (ctrl) in the following sections.

4.2.2. Phenotypic effects of *ftn-1* over-expression

In this section I examine the consequence of *ftn-1* OE on free iron levels, resistance to iron and peroxide toxicity on oxidative damage and ageing.

4.2.2.1. *ftn-1* OE does not reduce brood size

I first tested for toxic effects of the *wuEx187* transgene array. A standard test to determine if a transgene, mutation or agent has any negative effects on *C. elegans* are brood size measurements. The typical brood size of *C. elegans* wildtype worms at 20°C varies between 200 to 300 eggs per worm. Sick or stressed animals usually show a decrease in brood size. Therefore I measured the brood size of unmated wildtype, transgenic *ftn-1* OE and injection marker control hermaphrodites (see Materials & Methods, section 2.2.2.).

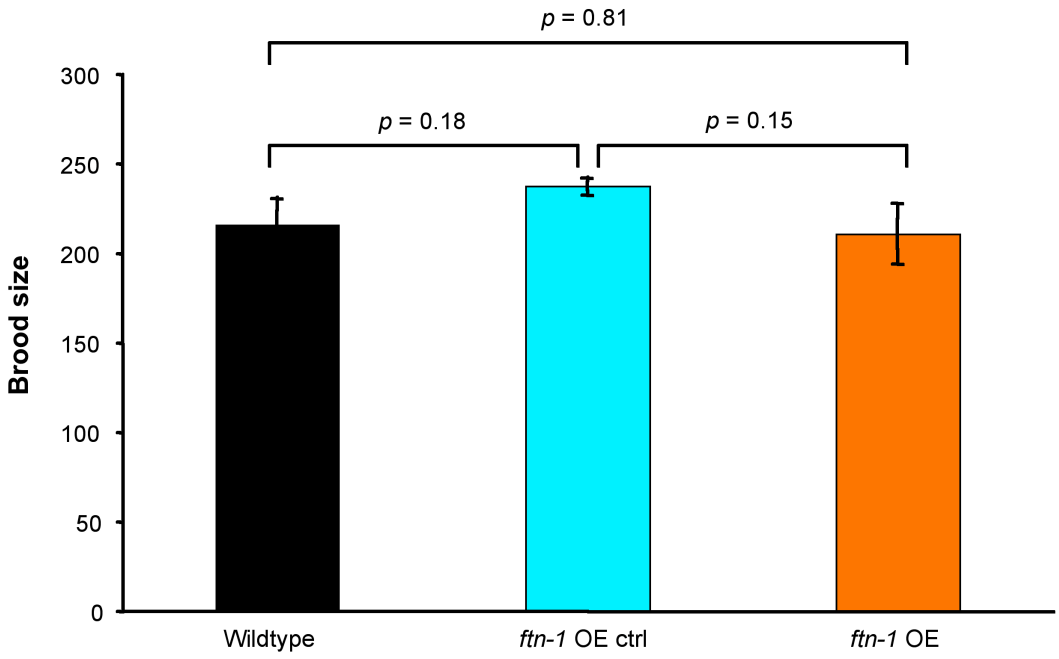


Figure 4.4: Brood size measurement of *ftn-1* over-expresser line and controls. Genotypes: N2 wildtype (n=20), *ftn-1* OE ctrl GA901 (*wuEx188 [coel::GFP]*) (n=10), *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*) (n=19). Each column represents the mean brood size of n animals, the error bars show the S.E.M. and the statistical analysis was performed with the Student's T-test.

The mean brood size of 20 individual wildtype worms (210 eggs) and 19 individual transgenic *ftn-1* over-expressing worms (216 eggs) were not significantly different (p=0.81). 10 worms of the transgenic line only injected with the *coel::GFP* plasmid had a mean brood size of 237, also not significantly different to wildtype (p=0.18) or *ftn-1* OE animals (p=0.15). Thus, brood size measurements did not reveal any negative effect of *ftn-1* over-expression or the *coel::GFP* marker.

4.2.2.2. *ftn-1* OE appears to reduce free iron levels

Since *ftn-1* encodes an iron storage protein, I expected that worms over-expressing this gene would have lower free iron levels (assuming that FTN-1 protein levels are actually elevated). To determine if this was the case, Mustafa Alam performed EPR measurements under my supervision (see Chapter 3, section 3.2.3.1. and 3.2.7.1.). Since large amounts of worms are necessary to get enough biomass for EPR measurements, we prepared an egg lay with *ftn-1* OE animals, egg prepped the synchronized F_1 generation and used the F_2 generation for the EPR measurements. This method to prepare the worms for the EPR sample was used to minimize the dilution of the sample and gain the highest percentage of transgenic animals possible (see Materials & Methods, section 2.2.2.1.).

Since the transgenic control has a lower transmission rate than the *ftn-1* OE line, the sample would mostly consist of wildtype animals in the F_2 generation. This would make it difficult to analyse the result of this measurement, therefore we did not include this line in the measurement. The results are presented in Figure 4.5 and Table 4.1.

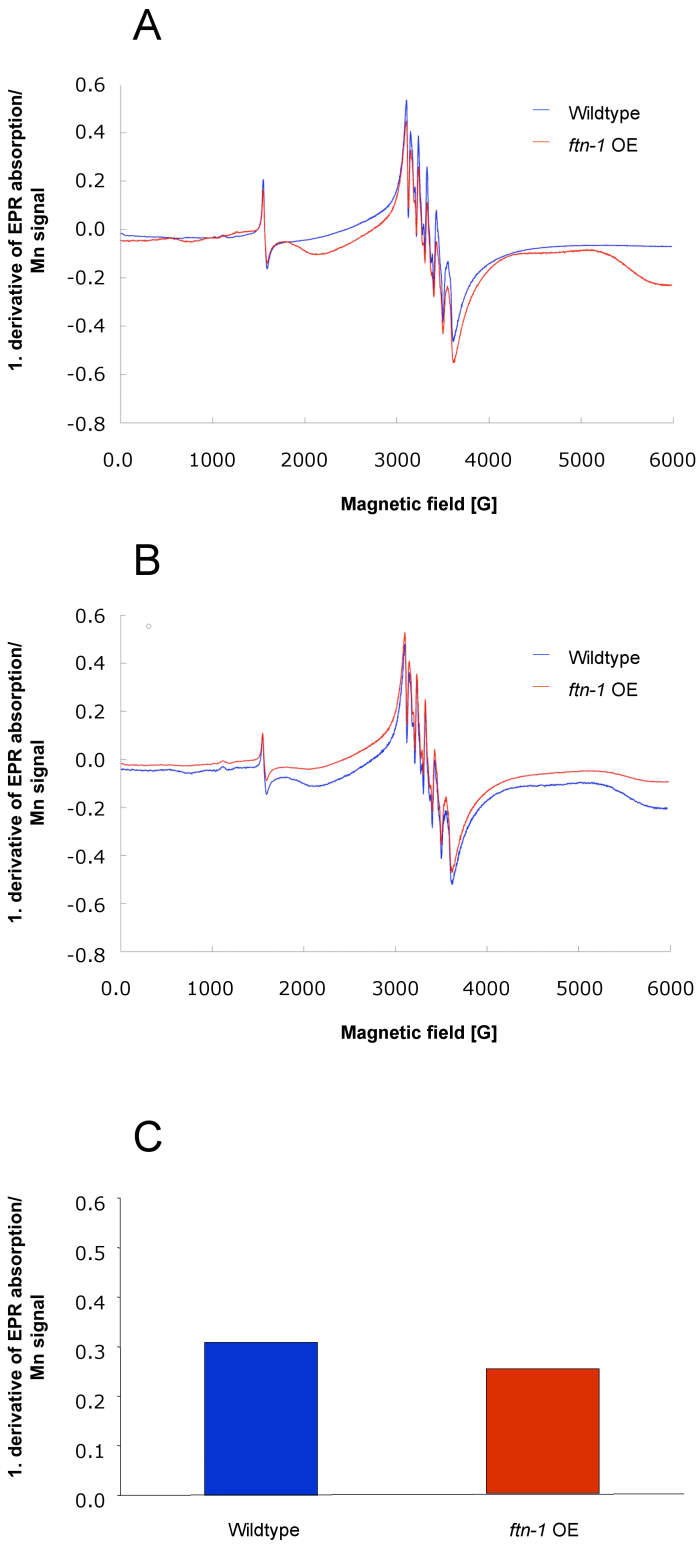


Figure 4.5: Effect of *ftn-1* OE on free iron levels in the worms. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*). **(A-B)** Individual EPR measurements with *ftn-1* OE and control animals. **(C)** Each column represents the mean of the two biological replicates. Since this measurement was performed only twice, it was not possible to do statistical analysis.

Table 4.1: Effect of *ftn-1* OE on free iron levels in the worms. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*). The numbers for each EPR measurement represent the length of the iron peak. Since this measurement was performed only twice, it was not possible to do statistical analysis. [n] Independent biological replicate number.

Strain	Length of iron peak (arbitrary units)	% change
Wildtype	**[C] 0.30** [1] 0.37 [2] 0.24	
ftn-1 OE	**[C] 0.25** [1] 0.30 [2] 0.19	**-16.7** -18.9 -20.8

Two independent biological replicates showed a reduction of free iron levels by 18.9% and 20.8% in the *ftn-1* OE line compared to wildtype animals. Combining the two trials gave an estimated 16.66% reduction in free iron levels in the transgenic animals. In the sample of the *ftn-1* over-expressing worms consisting of the F_2 generation, the frequency of transgenic worms will have been reduced due to the 65% transmission rate. The proportion of transgenic worms in the F_2 was not scored. It is therefore difficult to determine the precise reduction in free iron levels. However, 16.7% must be an underestimate of the degree to which free iron levels are reduced. More trials would be necessary to determine if and how statistically significant this apparent effect on free iron levels is.

4.2.2.3. *ftn-1* OE does not reduce oxidative damage levels

Treatment with the iron chelator DF did not reduce oxidative damage (see Chapter 3). To determine whether *ftn-1* OE is able to reduce oxidative damage levels, Dr Filipe Cabreiro and I measured protein carbonyl levels in the GA904 (*ftn-1* OE) strain (see Materials & Methods, section 2.2.13).

The biomass required for carbonylated protein measurements is less than for EPR ones. We were able to use the F_1 generation, instead of the F_2 generation, of the transgenic *ftn-1* OE and its genetic control, with the highest percentage of transgenic animals. Therefore we picked transgenic worms and let them lay eggs. An additional sample with N2 wildtype control was included to exclude any possible effects the *coel::GFP* marker might have on protein oxidation. The results are presented in Figure 4.6.

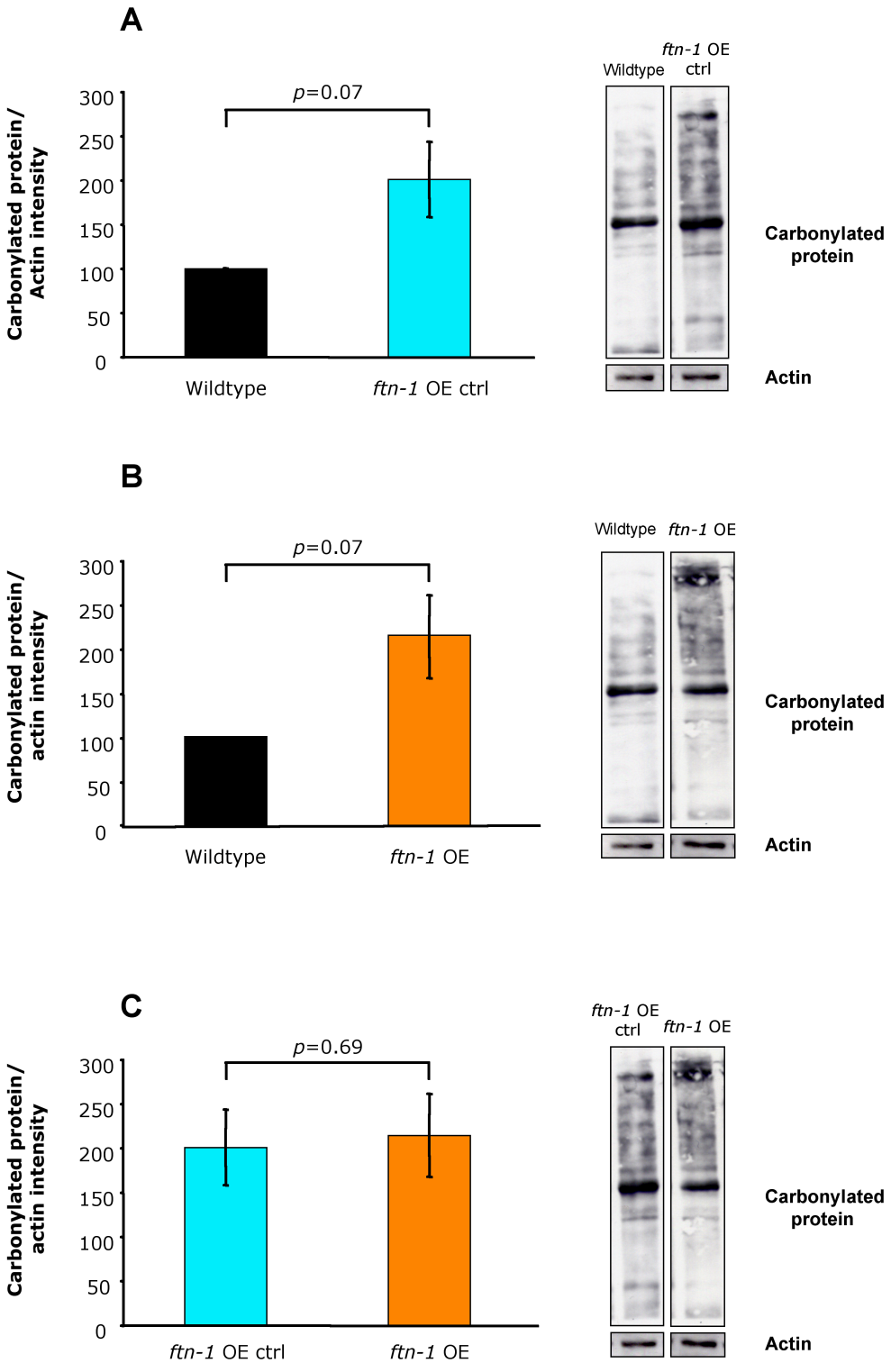


Figure 4.6: Effect of *ftn-1* OE on protein oxidation in *C. elegans*. Genotypes: N2 wildtype, *ftn-1* OE ctrl GA901 (*wuEx188 [coel::GFP]*), *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*). **(A-C)** Quantification of carbonylated protein against actin intensity with representative oxyblot on the right. Each column represents the mean of four biological replicates, two performed by Dr Filipe Cabreiro and two by myself, the error bars show the S.E.M. and statistical analysis was performed using the Student's T-test.

The *ftn-1* OE showed 6.7% higher levels of oxidized proteins than its transgenic control line, but this difference was not significant (p=0.69, Figure 4.6, C). This implies that the elevated *ftn-1* levels do not have an effect on overall protein oxidation levels in *C. elegans*.

Worryingly, the oxidized protein levels of the transgenic control and the *ftn-1* OE lines appeared markedly higher than wildtype levels (100% and 114.5%, respectively), although this difference did not quite attain statistical significance (p=0.07, Figure 4.6, A and B). Possible explanations include genetic background effects in the N2 cultivar used for the microinjection or an effect of the GFP marker (see Discussion, section 4.3.2.).

Overall, this data consistently implies that *ftn-1* over-expression does not reduce oxidative damage in *C. elegans*, as seen with DF supplementation. However, the apparently lower levels of protein carbonylation in the N2 line raises slight concerns that factors besides *ftn-1* OE might be increasing protein oxidation in both *ftn-1* OE and *ftn-1* OE control lines.

4.2.2.4. Effect of *ftn-1* OE on oxidative damage levels during iron stress

In the previous chapter (section 3.2.3.3) we showed that exposure to 15 mM iron (FAC) markedly increased protein oxidation. Since elevated *ftn-1* levels did not lower protein oxidation under standard culture conditions, I wanted to test whether the higher *ftn-1* levels can protect against the increase in oxidative damage caused by iron stress.

To this end, I exposed the transgenic animals to 15 mM iron, and Filipe Cabreiro and I performed oxyblot measurements. We again used the F_1 generation of transgenic animals and transferred the synchronized culture in the young adult stage to 15 mM FAC plates. The next day we collected the samples for protein carbonyl measurements. N2 wildtype and the transgenic line injected with the GFP marker were used as controls. The results are shown in Figure 4.7.

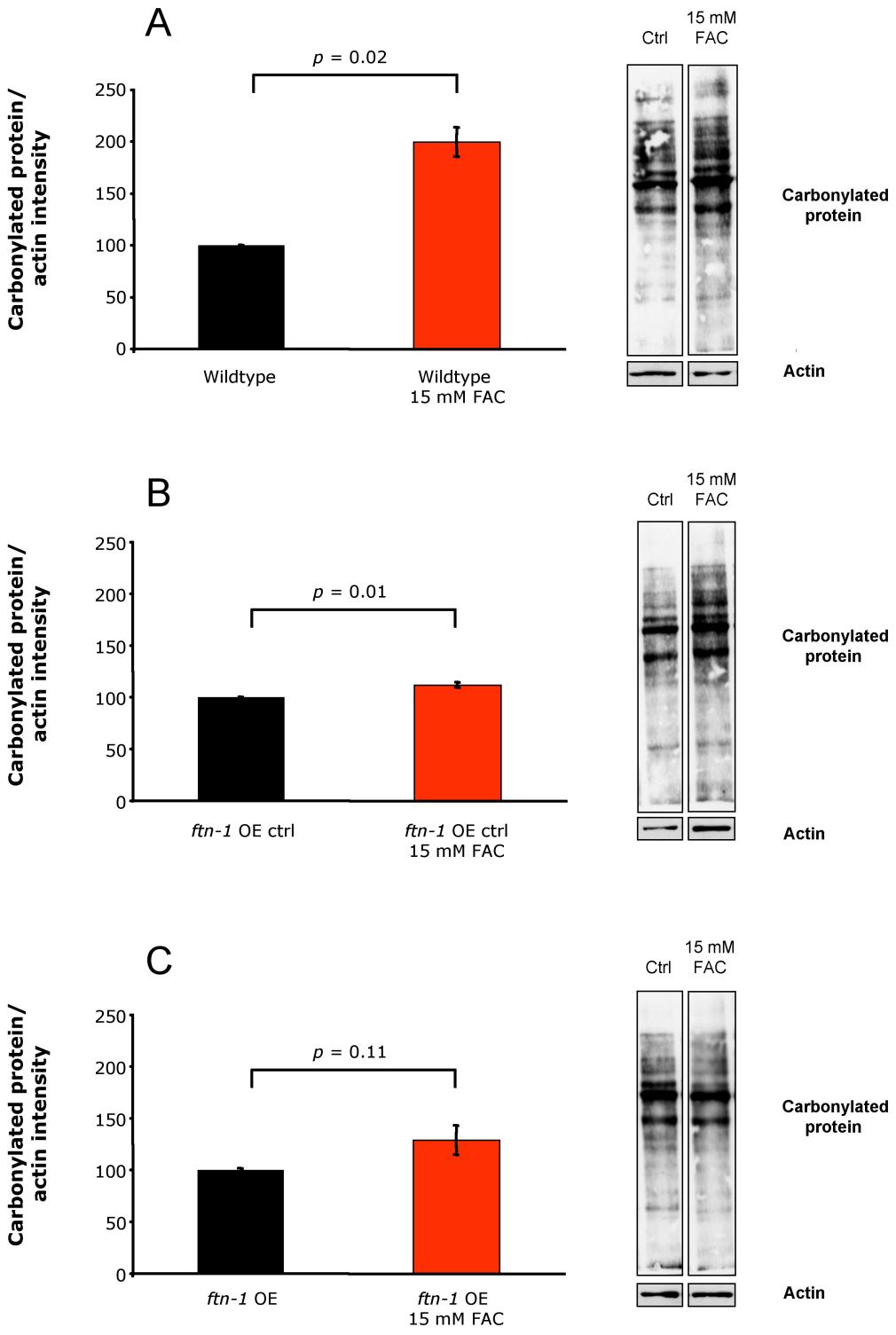


Figure 4.7: Effect of *ftn-1* OE on protein oxidation levels in *C. elegans* under iron stress. Genotypes: N2 wildtype, *ftn-1* OE ctrl GA901 (*wuEx188 [coel::GFP]*), *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*). **(A-C)** Quantification of carbonylated protein against actin intensity with representative oxyblot on the right. Each column represents the mean of four biological replicates, two performed by Dr Filipe Cabreiro and two by myself, the error bars show the S.E.M. and statistical analysis was performed using the Student's T-test.

As previously seen (Chapter 3, section 3.2.3.3.), wildtype animals showed elevated protein carbonyl levels upon 15 mM iron treatment (p=0.02, Figure 4.7, A). This was also true of the transgenic line injected only with the *coel::GFP* marker (p=0.01, Figure 4.7, B). But strangely, the magnitude of the increase in carbonyl levels was much higher in N2 (99.5%) than in the *ftn-1* OE control strain (11.8%). Compared to the *ftn-1* OE controls, transgenic animals over-expressing *ftn-1* showed a larger increase in oxidative damage (28.7%). However, this difference was not significant (p=0.11, Figure 4.7, C). If one takes the p values alone, this data seems to suggest that *ftn-1* over-expression protects against oxidative damage caused by excessive iron. However, if the magnitude of changes in carbonyl levels is considered as well, then it is difficult to draw firm conclusions from these trials.

4.2.2.5. *ftn-1* OE does not protect against iron supplement toxicity

Next, I tested whether over-expression of *ftn-1* protects against toxicity induced by high levels of iron. Here, the aim was, again, to verify that increased *ftn-1* expression leds to increased capacity to reduce free iron levels *in vivo*. I choose to expose the worms to 25 mM and 50 mM FAC, since both of these concentrations are toxic to *C. elegans* (see Chapter 3, section 3.2.5). *ftn-1* OE and wildtype animals were transferred as young adults to control plates or plates supplemented with FAC and subsequent survival monitored. Trials were conducted at 20°C and without FUdR (see Materials & Methods, section 2.2.6.1). The results are presented in Figure 4.8 and Table 4.2.

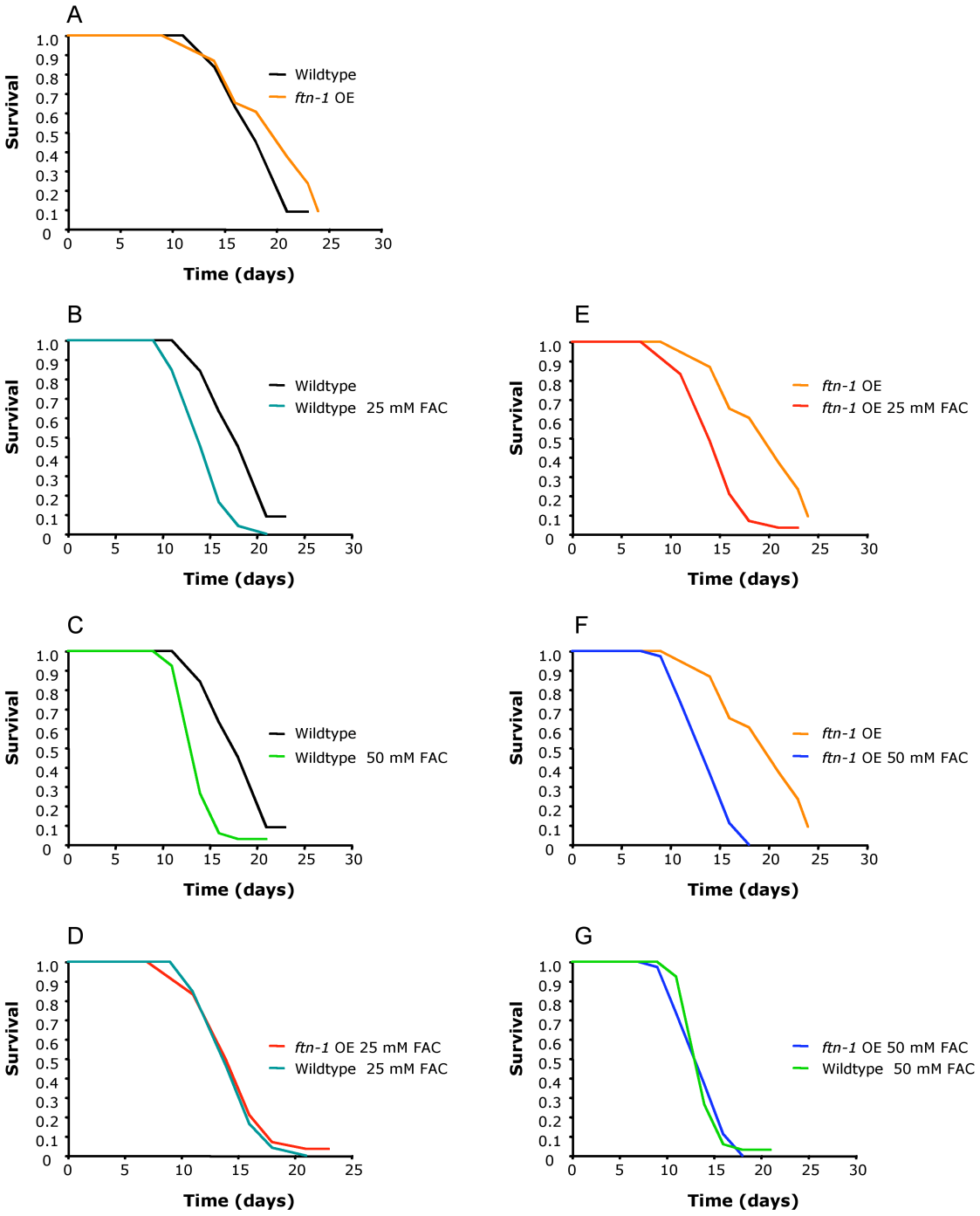


Figure 4.8: Effect of *ftn-1* OE on survival under iron stress. The trials were performed at 20˚C without FUdR. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*). **(A-G)** Survival curves of *ftn-1* OE worms on 25 and 50 mM FAC compared to control animals. Statistical analysis is presented in Table 4.2.

Table 4.2: Effect of *ftn-1* OE on survival under iron stress. The trials were performed at 20˚C without FUdR. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*). *p*, probability of being the same as specified control (log rank). [n] biological replicates number. The last two columns compare the two strains with the same FAC treatment.

Strain	**FAC (mM)**	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* genetic ctrl**	***p vs.* genetic ctrl**	**% *vs.* treatment**	***p vs.* treatment**
Wildtype	Ctrl	21/20	18.3				
Wildtype	25	30/10	14.9	-18.6	<.0001		
Wildtype	50	36/6	14.4	-21.3	<.0001		
ftn-1 OE	Ctrl	20/14	19.8			+8.2	0.11
ftn-1 OE	25	28/5	15.1	-23.7	<.0001	+ 1.3	0.60
ftn-1 OE	50	30/8	14.1	-28.8	<.0001	–2.1	0.32

Wildtype and *ftn-1* OE worms both showed a significant reduction in life span upon treatment with 25 mM and 50 mM iron (see Figure 4.8 B, C, E and Table 4.2). Comparing survival of wildtype and *ftn-1* OE animals on the same iron concentration did not reveal any significant difference (Figure 4.8 D, G). Therefore this single trial did not show a resistance to iron stress in worms over-expressing *ftn-1* at the iron concentrations tested here. Possibly this is because at the iron levels tested, the carrying capacity of ferritin becomes saturated. If so then exposure to lower iron concentrations, such as 15 mM, might reveal a protective role of *ftn-1* over-expression during iron stress that could not be detected at the higher concentrations. However, I decided to not further pursue this in favour of oxidative stress tests.

This preliminary life span data showed an 8.2% increase in life span of *ftn-1* OE animals compared to wildtype ones, but this difference was not statistically significant (p=0.11, Figure 4.8 A). Further replicates are necessary to test the effect of elevated *ftn-1* levels in *C. elegans* ageing (see section 4.2.2.7.)

4.2.2.6. *ftn-1* OE increases resistance to *tert*-butyl hydroperoxide (TBH)

If *ftn-1* OE lowers iron, it should, like 100 µM deferoxamine, increase resistance to peroxide toxicity.

I therefore tested the effect of *ftn-1* OE on resistance to TBH. I picked transgenic *ftn-1* OE and injection marker control worms by selecting worms carrying the GFP marker to plates containing 10 mM TBH. As a further control N2 wildtype worms were added (see Materials & Methods, section 2.2.6.2.). The results are presented in Figure 4.9 and Table 4.3.

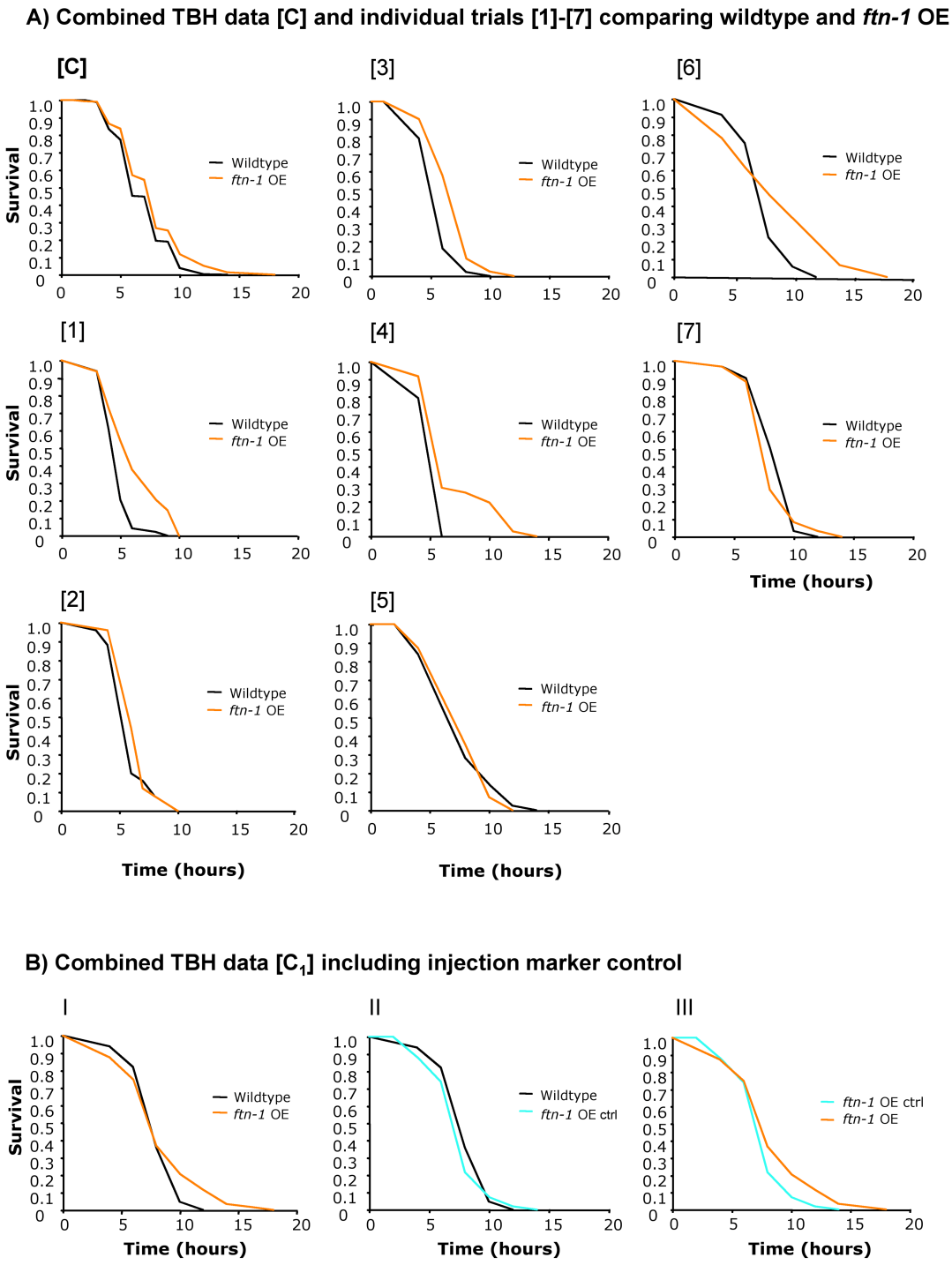


Figure 4.9: Effect of *ftn-1* OE on peroxide toxicity in *C. elegans*. The trials were performed at room temperature on 10 mM TBH plates. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*) and the injection marker control *ftn-1* OE ctrl GA901 (*wuEx188 [coel::GFP]*). **(A)** Shows the combined survival curve **[C]** of seven biological replicates comparing the *ftn-1* OE with wildtype worms and the individual trials **[1-7]**. **(B)** Shows the combined survival curves **[C_1]** of two biological replicates including the *ftn-1* OE ctrl. Statistical analysis is presented in Table 4.3.

Table 4.3: Effect of *ftn-1* OE on peroxide toxicity in *C. elegans*. The trials were performed at room temperature on 10 mM TBH plates. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*) and the injection marker control *ftn-1* OE ctrl GA901 (*wuEx188 [coel::GFP]*). *p*, log rank test. [n] biological replicates number.

Strain	Deaths/ censored	Mean life span (hours)	% *vs.* wildtype	*p vs.* wildtype	% *vs. ftn-1* OE ctrl	*p vs. ftn-1* OE ctrl
Wildtype	**[C] 320/11**	**7**				
	[1] 49/0	4.9				
	[2] 25/0	6.2				
	[3] 45/7	5.9				
	[4] 29/0	5.6				
	[5] 43/4	7.7				
	[C_1] 129/0	**8.3**				
	[6] 68/0	7.9				
	[7] 61/0	8.8				
ftn-1 OE ctrl	**[C_1] 111/5**	**7.9**	**-4.8**	**0.11**		
	[6] 57/0	7.6	-3.8	0.62		
	[7] 54/5	8.1	-8	0.02		
ftn-1 OE	**[C] 299/16**	**7.7**	**+10.9**	**0.0002**		
	[1] 48/0	6.3	+28.6	0.0001		
	[2] 25/0	6.6	+6.5	0.35		
	[3] 40/10	7.2	+22	0.0002		
	[4] 36/0	7.3	+30.4	0.002		
	[5] 30/6	7.8	+1.3	0.96		
	[C_1] 120/0	**8.7**	**+4.8**	**0.06**	**+10.1**	**0.005**
	[6] 60/0	9	+13.9	0.006	+18.4	0.005
	[7] 60/0	8.5	-3.4	0.19	+4.9	0.43

To exclude any possible effects of the *coel::GFP* marker, I included the transgenic line carrying the GFP marker, without the extra copies of *ftn-1*, in two trials ([6] and [7]). Overall, the control strain showed a 4.8% reduction in survival upon TBH treatment compared to wildtype animals (7.9 hours *vs.* 8.3 hours, respectively, Figure 4.9 II), but this difference was not significant (p=0.11). However, one of the two trials ([7]) showed a significant decrease upon TBH treatment compared to wildtype (8.8 hours *vs.* 8.1 hours, p=0.02, respectively). Trial six showed a 3.8% decrease in survival of the transgenic control line to wildtype animals, but this difference was not statistically significant (p=0.62). This data suggests that the transgenic line carrying the *coel::GFP* plasmid might be more sensitive to oxidative stress induced via TBH than wildtype animals. Further replicates would be necessary to establish this with certainty.

In four out of seven TBH stress assays, transgenic *ftn-1* OE worms showed a significantly higher survival than wildtype worms (6.3-9 hours *vs.* 4.9-7.9 hours, respectively, [1], [3-4], [6]). In the remaining three trials, worms carrying the *wuEx187* transgene, appeared to show a higher survival rate, but this difference was not significant to wildtype animals (6.5%, p=0.35 and 1.3%, p=0.96, respectively, [2] and [5]), and once the over-expresser showed a decreased survival by 3.4%, but this difference was again not significant (p=0.19, [7]).

The combined data comparing the *ftn-1* OE with its genetic control *ftn-1* OE ctrl ([C_1]) showed also an increased resistance of *ftn-1* OE worms to stress induced via TBH (8.7 hours *vs.* 7.9 hours, p=0.005, respectively, Figure 4.9, III). The data combines one trial showing an 18.4% resistance to

oxidative stress compared to the *ftn-1* OE ctrl (p=0.005, [6]) and one showing 4.9% higher survival than the GFP control animals, but this difference was not significant (p=0.43, [7]).

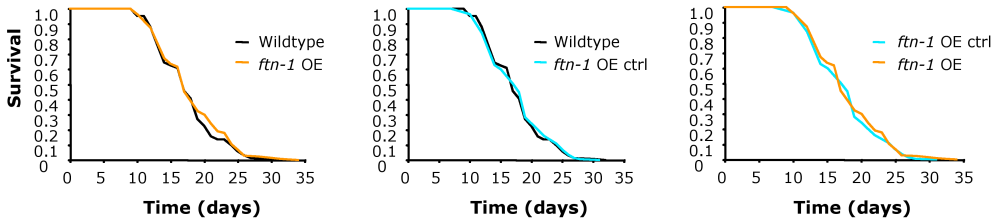
Trial seven reduced the overall mean survival of *ftn-1* OE to non-significant levels compared to wildtype animals in the two trials including the GFP marker control ([C_1]: 8.7 hours *vs.* 8.3 hours, p=0.06, respectively, Figure 4.9, I). However, trial seven is the only trial showing a decrease of survival. Since four other trials showed the contrary result and two showed no significant difference, but at least a higher survival rate, this trial seems to be an exception.

Overall, the data shows that *ftn-1* over-expression protects against TBH. This is consistent with reduced levels of free iron and of the Fenton chemistry in the *ftn-1* OE line.

4.2.2.7. *ftn-1* OE has no effect on ageing

If, under standard culture conditions, levels of free iron limit life span through their effects on Fenton chemistry, then over-expression of *ftn-1* would be expected to increase life span. To test this, I compared survival in *ftn-1* OE control and N2 population. Transgenic worms were picked at the L4 stage expressing GFP in the six coelomocytes. The worms were transferred to fresh plates every day the first week of adulthood to exclude offspring (see Materials & Methods, section 2.2.4.). The results are presented in Figure 4.10 and Table 4.4.

A) Combined life span data [C]



B) Life span data of individual trials [1]-[4]

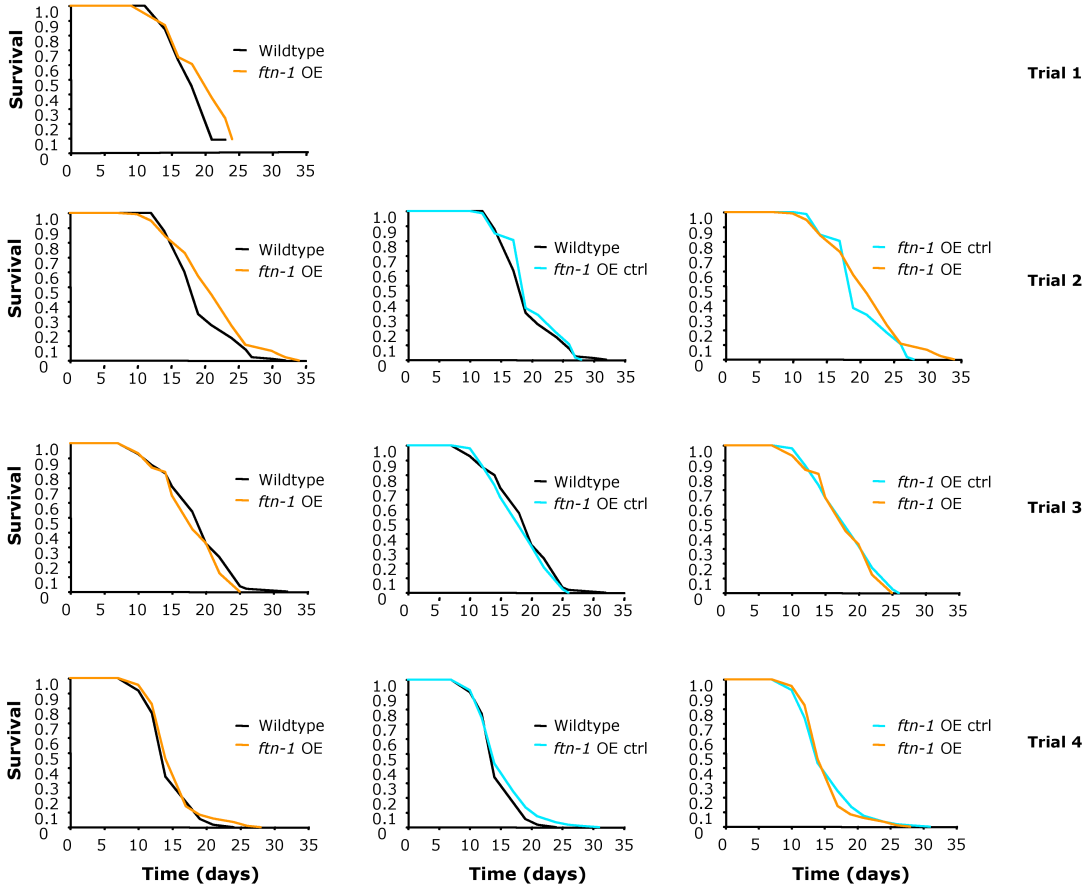


Figure 4.10: Effect of *ftn-1* OE on life span in *C. elegans*. The trials were performed at 20°C without FUdR. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*) and the injection marker control *ftn-1* OE ctrl GA901 (*wuEx188 [coel::GFP]*). **(A)** Shows the combined life span data **[C]** of four biological replicates comparing the *ftn-1* OE with wildtype worms and the transgenic control. **(B)** Shows the individual biological replicates. Statistical analysis is presented in Table 4.4.

Table 4.4: Effect of *ftn-1* OE on life span in *C. elegans*. The trials were performed at 20°C without FUdR. Genotype: N2 wildtype, *ftn-1* OE GA904 (*wuEx187 [Pftn-1::ftn-1::ftn-13'UTR + coel::GFP]*) and the injection marker control *ftn-1* OE control GA901 (*wuEx188 [coel::GFP]*). *p*, log rank test. [n] biological replicates number.

Strain	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* wildtype**	***p vs.* wildtype**	**% *vs.* *ftn-1* OE ctrl**	***p vs. ftn-1* OE ctrl**
Wildtype	**[C] 306/149**	**17.5**				
	[1] 21/20	18.3				
	[2] 94/33	19.6				
	[3] 66/59	18.9				
	[4] 125/37	14.9				
ftn-1 OE ctrl	**[C] 270/118**	**17.7**	**+1.1**	**0.64**		
	[2] 66/55	20.2	+3	0.26		
	[3] 80/34	18	-4.8	0.34		
	[4] 124/29	15.7	+5.4	0.05		
ftn-1 OE	**[C] 266/164**	**18**	**+2.9**	**0.29**	**+1.7**	**0.58**
	[1] 20/14	19.8	+8.2	0.11		
	[2] 58/62	21.4	+9.2	0.02	+6	0.19
	[3] 67/58	18.3	-3.2	0.36	+1.7	0.97
	[4] 121/30	15.6	+4.7	0.08	-0.6	0.78

The transgenic line injected only with the *coel::GFP* plasmid showed once, in trial four, a significantly longer life span than wildtype animals (15.7 days *vs.* 14.9 days, respectively, p=0.05). However, two further trials showed no difference in life span to wildtype animals (17.7 days *vs.* 17.5 days, respectively, p=0.64). This supports the view that the *coel::GFP* marker alone has no effect on *C. elegans* life span.

The transgenic *ftn-1* OE line showed also once a significantly longer life span than wildtype (21.4 days *vs.* 19.6 days, respectively, p=0.02), but this was not the case in three further replicates (19.8 days *vs.* 18.3 days, p=0.11; 18.3 days *vs.*18.9 days, p=0.36; 15.6 days *vs.* 14.9 days, p=0.08, respectively).

Furthermore, the comparison of the *ftn-1* OE line with its transgenic control line did not show once, in three biological replicates, a significant difference to each other (18 days *vs.* 17.7 days, p=0.58, respectively).

In trial four, all mean life spans of the three different strains are shorter than usually expected for wildtype animals at 20°C. Since all three strains are short-lived, a background effect or spontaneous mutation can be ruled out. Therefore it seems that other circumstances, such as culture conditions, caused a decrease in mean life span of all three strains in this one trial.

Overall, this data shows that neither the GFP marker nor the *ftn-1* over-expression have an effect on life span in *C. elegans*. Thus, as with 100 µM deferoxamine, a treatment that lowers free iron levels and increases resistance to oxidative stress, has no effect on life span. This again suggests that free iron and the chemistry that it promotes are not imitating for life span in *C. elegans* under standard culture conditions.

4.3. Discussion

4.3.1. The effect of *wuEx187* on free iron levels

The EPR measurements detected a 16.6% reduction in free iron in the *ftn-1* OE line compared to wildtype animals. However, since we conducted this measurement only twice, it is not possible to determine if and how significant this decrease in free iron levels was. Also, the sample used for EPR measurement consisted of the F_2 generation of transgenic animals, and since the *wuEx187* transgene array is not integrated, the sample included a proportion of worms that did not inherit *wuEx187*. The proportion of F_2 worms that carried the *wuEx187* transgene was not measured. However, one may estimate very approximately the proportion of the F_1s on the basis of the 65% transmission rate. Of the F_2, 65% of 65%, or 42% should have been transgenic. If 42% of worms in the EPR sample were transgenic, and there was an overall reduction of free iron levels of 16.6%, then this predicts that free iron levels are reduced by 39.6% in the transgenic worms. One possible way to verify the reduction in free iron levels would be to cross *wuEx187* into a *Pftn-1::GFP* strain and see whether it reduces GFP levels. However, the fact that *ftn-1* OE worms show an apparent reduction in the free iron EPR signal despite the dilution of transgenic animals supports the view that *ftn-1* over-expression reduced free iron levels in *C. elegans*. Furthermore, the oxidative stress resistance of the *ftn-1* OE line further supports this view.

4.3.2. The effect of *wuEx187* on oxidative damage levels

We saw no reduction in oxidative damage to proteins after *ftn-1* over-expression. Since a sample for carbonylated protein measurements requires large numbers of worms, similar dilution problems occurred as already mentioned for EPR measurements. However the sample was less diluted as in the case of EPR measurements, since the biomass necessary was not as large as for the iron detection and therefore it was possible to use the F_1 generation. What does this apparent lack of an effect signify? One possibility is that FTN-1 levels are not elevated, despite the increased *ftn-1* mRNA levels. Here one may recall that *ftn-1* is posttranscriptionally regulated. However, the other impacts of elevated *ftn-1* expression, such as higher resistance to oxidative stress, argue against this conclusion. Thus, these results suggest that reducing free iron levels in young adults has no effect on levels of protein oxidation. Thus argue that in this context free iron and, by inference, the Fenton reaction, do not influence steady state levels of protein oxidation. However, it remains possible that free iron levels do affect levels of other kinds of molecular damage, and that protein oxidation and/or other forms of molecular damage are affected by free iron levels in other contexts, e.g. in later life, or under stress.

The oxidative damage results contained an oddity. While protein carbonyl levels did not differ between *ftn-1* OE and *coel::GFP* control, levels in both appeared elevated relative to wildtype controls showing a 100-114.5% increase, although this difference did not quite reach statistical significance due to high variation between the individual blots (p=0.07). The basis of this

difference is unclear. Since both transgenic lines have higher levels of protein oxidation than wildtype, *ftn-1* over-expression can be excluded as a cause.

One possible explanation is a background effect in the N2 cultivar used for the generation of both transgenic lines, leding to elevated oxidative damage to proteins. Another thing that the transgenic lines have in common is the GFP marker. Could this led to increased protein oxidation? Recently a poster was published by Lin et al. reporting that the chromophores of fluorescent proteins, such as GFP, are formed in an autocatalytic reaction using O_2 and generating H_2O_2 (Lin M. Z. et al., 2010). Considering this finding, it is possible that the transgenic lines carrying the *coel::GFP* marker actually have higher levels of H_2O_2, causing elevated protein oxidation. However, given that GFP expression is limited in these lines to a very small number of cells, the six coelomocytes, it seems unlikely that GFP is the cause of the effect on protein oxidation.

Another possibility is that the UV light of the epifluorescence microscope, used to identify the transgenic animals, caused oxidative damage in these animals. UV light is well known to cause oxidative damage in biological systems, and a recent report analysed different wavelengths of light and their effect on free radical production. Here, light of 280-1600 nm can induce various levels of ROS formation (Zastrow et al., 2009). Since the excitation and emission wavelength for GFP is 395 and 509 nm respectively, it is possible that both transgenic lines have higher levels of oxidative damage due to the fluorescence selection of their GFP marker. However, given that it was the F_1 progeny of transgenic animals exposed to UV whose protein carbonyl levels were increased, any such UV damage would have to have

occurred in embryos, oocytes and sperm, and pre-oocytes. Given that the vast preponderance of the protein present in the F_1 samples tested must have been synthesized after the UV exposure, and given the magnitude of the difference in protein oxidation levels, UV seems unlikely to be the cause of this oddity.

4.3.3. The effect of *wuEx187* on resistance to oxidative stress

Overall, the *ftn-1* OE line is significantly more resistant to oxidative stress induced via TBH than wildtype or *coel::GFP* control animals. This result supports the view that the *ftn-1* over-expression is reducing free iron levels required for the hydroxyl radical generation via the Fenton reaction. Additionally, worms treated with the iron chelator DF showed increased resistance to oxidative stress too (see Chapter 3, section 3.2.8.) and wildtype animals with reduced *ftn-1* levels showed increased sensitivity to oxidative stress (see Chapter 3, section 3.2.2.), confirming the role of *ftn-1* in oxidative stress.

4.3.4. The effect of *wuEx187* on organismal ageing

Despite conferring resistance to oxidative stress, *ftn-1* OE did not increase *C. elegans* life span. This finding is consistent with earlier iron chelator studies in that in both cases lowering free iron increases resistance to oxidative stress without having any effect on life span. These findings argue against the oxidative damage theory of ageing.

This conclusion is consistent with many recent findings questioning the role of oxidative damage in ageing. For instance, the drug antimycin A increases generation of $O_2\bullet^-$ in *C. elegans* mitochondria (Senoo-Matsuda et al., 2001) while slightly increasing life span (Dillin et al., 2002) and long-lived *daf-2* mutants show an increase in ROS production (Brys et al., 2007). Furthermore, treatment with a drug inhibiting glycolysis, 2-deoxy-D-glucose (DOG), mimicking a dietary glucose restriction, caused elevated ROS production, resistance to oxidative stress and increase in life span (Schulz et al., 2007). Studies like these support the view of ROS as important signalling molecules. This assumption is based on findings indicating that ROS induces stress responses leding to life span extensions (Connor et al., 2005; Kharade et al., 2005; Loh et al., 2009; Vanfleteren, 1993; Zarse et al., 2007). It was also recently shown that increased oxidative stress can promote longevity (Ristow and Zarse, 2010). These results could imply the opposite effect of oxidative damage on life span than the predicted one. Likewise, deletion of *sod-2* increases oxidative damage levels and stress sensitivity and either has no effect on life span (Doonan et al., 2008; Honda et al., 2008; Yang et al., 2007; Yen et al., 2009) or even increases life span in a *daf-2* mutant background (Van Raamsdonk and Hekimi, 2009; Yang et al., 2007).

4.3.5. Conclusion

Numerous genes influence ROS production, such as those encoding the antioxidants SOD and catalases (see Introduction, section 1.4.9 and 1.4.10.). Here, I investigated the role of the iron storage protein gene *ftn-1* and

found it to protect against oxidative stress, without affecting *C. elegans* life span.

Different approaches, such as DF or FAC treatment, caused decreased and increased oxidative stress, respectively, and while high levels of iron did reduce life span, reduced levels of iron did not have the opposite effect. These results argue against the oxidative damage theory of ageing, and further support the scepticism about this theory generated by a number of recent studies (see Introduction, section 1.5.).

Chapter 5: The effect of *sir-2.1* over-expression on *C. elegans* life span in the transgenic line LG100

5.1. Introduction

The controversies surrounding sirtuins and their role in ageing led us to seek to verify the effects on ageing of *sir-2.1* over-expression in *C. elegans* (see main Introduction, section 1.6.2. and 1.6.3.). The claim that *sir-2.1* over-expression increases life span in *C. elegans* is largely based on studies using two *sir-2.1* over-expressing strains (Berdichevsky et al., 2006; Tissenbaum and Guarente, 2001; Viswanathan et al., 2005).

5.1.1. A chromosomal duplication including *sir-2.1* increases life span

The idea that *sir-2.1* might promote longevity in *C. elegans* as well as in yeast has its origin from a screen of *C. elegans* strains with duplications of chromosomal regions (Tissenbaum and Guarente, 2001). This screen was performed in the laboratory of L. Guarente at the Massachusetts Institute of Technology, USA, where the role of sirtuins in yeast ageing was discovered (see Introduction, section 1.6.2.1.). The strain DR1786, carrying the large free duplication of chromosome IV, *mDp4*, that contains the *sir-2.1* locus, was one of only four duplication strains showing a significant increase in life span. The other three duplication strains KR1732, RW6011 and SP125 did not include *sir-2.1* in the duplicated regions (for further details see 5.2.7.). Interestingly, MT7070 and DR907, similar duplications to *mDp4*, but not including the *sir-*

2.1 locus, did not show an increase in life span. This suggested the possibility that a single extra copy of *sir-2.1* is sufficient to increase *C. elegans* life span. Duplication strains containing the other three s*ir-2.1* homologues, *sir-2.2 sir-2.3* (SP117 *mnDp10,* SP116 *mnDp9,* SP75 *mnDp25)* and *sir-2.4* (KR1108 *hDp8,* KR1110 *hDp11,* KR1112 *hDp9,* KR1236 *hDp2,* KR1284 *hDp15,* KR1280 *hDp13,* KR1548 *hDp22,* KR1282 *hDp16,* KR1293 *hDp12*, KR1725 *hDp44*, KR1722 *hDp52*, KR1704 *hDp68,* KR1732 *hDp48*) did not show any repeatable increase in life span.

5.1.2. Creation of LG100

After this finding, a *sir-2.1* over-expressing *C. elegans* strain was generated using microinjection (Tissenbaum and Guarente, 2001). For this a 2.2kb PCR fragment containing the complete *sir-2.1* sequence plus 400bp upstream and 300bp downstream of the predicted open reading frame was co-injected with the dominant *rol-6(su1006)* transformation marker. It was reported that three independent transgenic lines, containing transgene arrays *geEx1*, *geEx2* or *geEx3*, showed an increase in life span. Integrated lines were created from *geEx1* (*geIn1*, *geIn2*) and *geEx2* (*geIn3*) using gamma irradiation, and these reportedly showed similar effects on life span to their extrachromosomal predecessors.

In the strain LG100 *geIn3 [sir-2.1, rol-6(su1006)]*, a large increase in *sir-2.1* gene copy number was indicated by Southern blotts. The reported mean life span was 27.5 days, an increase of 50.3% relative to the *rol-6* control strain. The life span increase of the *sir-2.1* over-expresser LG100 proved to be *daf-16* dependent as shown by crossing *geIn3* into a *daf-*

16(mgDf50) mutant background. Furthermore, the *sir-2.1* over-expressing lines *geEx2* and *geEx3* increased dauer formation in *daf-1* and *daf-4* mutant backgrounds. The *geEx2* line had a reported life span increase of 49.7%, whereas *geEx3* showed 22.4% extension in life span. The degree of enhancement of dauer formation correlated with the respecting life span extension of these transgenic lines. *daf-4(m63); geEx2* showed 48% (n=229) and *daf-1(m40); geEx2* 29.5% (n=285) dauer formation whereas *daf-4(m63); geEx3* showed only 29% and *daf-1(m40); geEx3* 13.0% compared to control animals *daf-4(m63)* (n=981) 10% and *daf-1(m40)* (n=670) 0.6%. This led to the conclusion that the *sir-2.1* transgene arrays have dose dependent effects on Daf-c and Age, and that it reduces insulin/IGF signalling (IIS) and/or activates DAF-16 (Tissenbaum and Guarente, 2001).

Since the integrated line *geIn3* (strain LG100 *geIn3 [sir-2.1, rol-6(su1006)]*) showed the largest increase in life span, was a focus of study in the 2001 report and was available via the Caenorhabditis Genetics Center (CGC), I used this strain for the experiments presented in this chapter.

In this chapter and the one that follows I describe a series of studies aimed at verifying the effect of *sir-2.1* over-expression on *C. elegans* life span. Several factors, in particular, motivated this work. Firstly, a previous member of the Gems lab (J.J. McElwee), acting on a personal communication at the 2005 International *C. elegans* meeting, backcrossed LG100 and found that its longevity disappeared (see below). Secondly, work in the Partridge laboratory at UCL found that *dSir2* did not increase life span in *Drosophila* (see Chapter 6, Discussion), contrary to a published report (Rogina and Helfand, 2004).

5.2. Results

5.2.1. The *sir-2.1* over-expressing line LG100 is long-lived

At the outset of this project, I first wanted to reconfirm that LG100 shows an increased life span. LG100 was obtained prior to my arrival at UCL by J. J. McElwee. Life span measurements were performed as described in Materials & Methods (section 2.2.4) using N2 wildtype and a *rol-6* transformant (GA707) as non-long-lived, negative controls. Life span trials were performed at 20˚C. One day before the start of trials, FUdR was applied to the plates to a final concentration of 10 µM or 40 µM FUdR to prevent progeny production. L4 stage nematodes were transferred to FUdR plates at the start of each trial. The results are shown in Figure 5.1 and Tables 5.1.

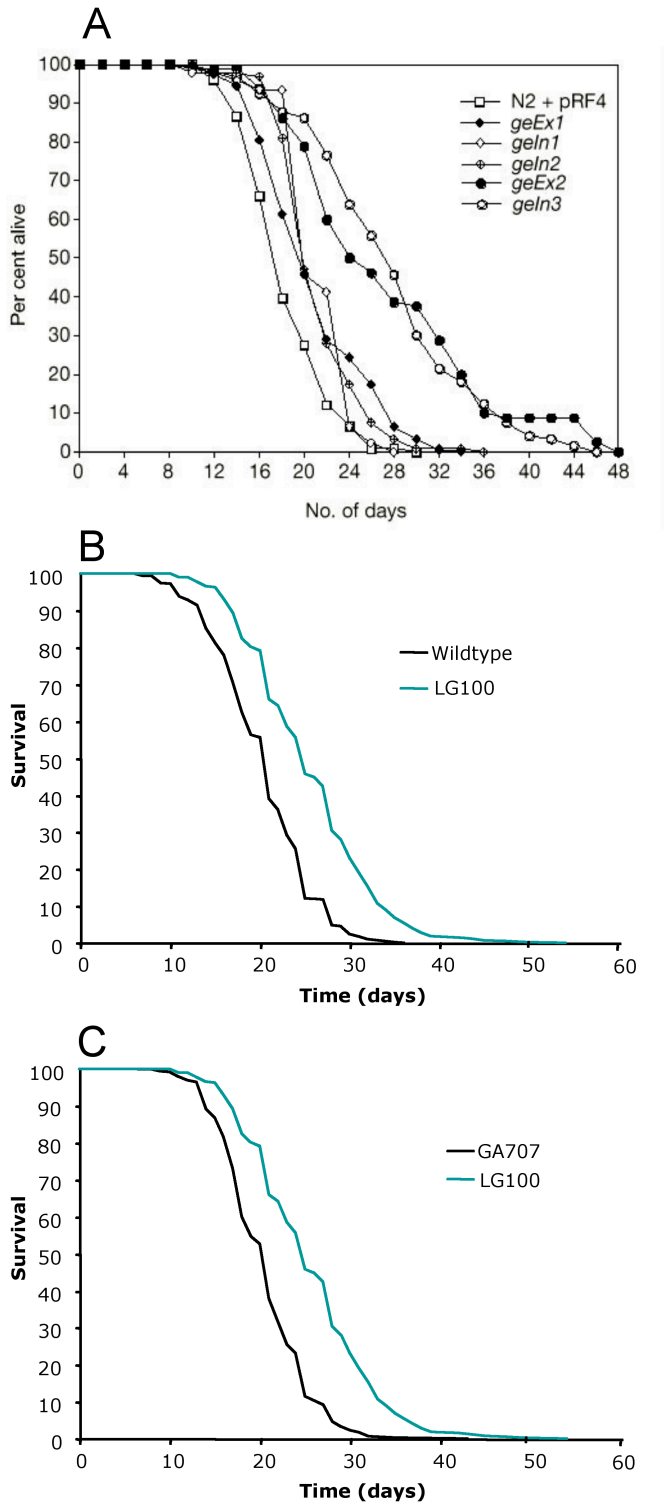


Figure 5.1: Life span effect of *sir-2.1* over-expression in LG100. **(A)** Original LG100 life span curves reproduced from (Tissenbaum and Guarente, 2001). **(B-C)** Genotypes: N2 wildtype, GA707 *wuEx166 [rol-6(su1006)],* LG100 *geIn3 [sir-2.1 rol-6(su1006)]*. LG100 life span compared to wildtype animals and a *rol-6* control strain. The life span curves represent the combined data of six independent biological replicates. The statistical analysis is presented in Table 5.1.

Table 5.1: Life span effect of *sir-2.1* over-expression in LG100. The trials were performed at 20˚C, with FUdR at 10 µM (trial 4-6) or 40 µM (trial 1-3). Genotypes: N2 wildtype, GA707 *wuEx166 [rol-6(su1006)],* LG100 *geIn3 [sir-2.1 rol-6(su1006)].* [C] combined data, *p*, probability of being the same as specified control (log rank). [n] Independent biological replicate number.

Strain	Deaths/ censored	Mean life span (days)	% *vs.* wildtype	*p vs.* wildtype	% *vs.* GA707	*p vs.* GA707
Wildtype	**[C] 598/228**	**20.4**				
	[1] 84/43	18.9				
	[2] 79/48	19.9				
	[3] 62/63	22.6				
	[4] 86/34	19				
	[5] 164/9	21				
	[6] 123/31	20.1				
GA707	**[C] 440/226**	**20.5**	**+0.5**	**0.66**		
	[1] 81/49	21	+11.1	0.01		
	[2] 96/29	21.8	+9.5	<.0001		
	[3] 75/50	23.6	+4.4	0.13		
	[4] 63/53	17	-10.5	0.0007		
	[5] 44/19	18.5	-11.9	0.003		
	[6] 81/79	19.2	-4.5	0.10		
LG100	**[C] 678/115**	**25.6**	**+25.5**	**<.0001**	**+24.9**	**<.0001**
	[1] 91/17	26.6	+40.7	<.0001	+26.7	<.0001
	[2] 119/7	31.8	+59.8	<.0001	+45.9	<.0001
	[3] 116/6	26.3	+16.4	0.0001	+11.4	0.002
	[4] 106/29	21.9	+15.3	<.0001	+28.8	<.0001
	[5] 146/7	24.9	+18.6	<.0001	+34.6	<.0001
	[6] 100/49	21.1	+5	0.02	+9.9	0.004

Six independent biological replicates confirmed the reported claim that the high copy *sir-2.1* over-expressing strain LG100 is long-lived relative to wildtype and *rol-6* controls (ranges: 21.1-31.8 days *vs.* 17-23.6 days and 18.9-22.6 days, respectively). In most cases statistical significant levels reached $p<.0001$ (log rank test). Combined data showed an increase in mean life span in LG100 of 25.5% and 24.9% relative to N2 and *rol-6*, respectively. Increase in mean life span ranged from 5% to 59.8%.

The *rol-6* control strain GA707 showed a larger variation in life span than wildtype animals. In trial [1] and [2], *rol-6* animals were significantly longer-lived than wildtype ($p=0.01$, <.0001), in trials [3] and [6], there was no detectable difference ($p=0.13$, 0.10) and in trials [4] and [5] *rol-6* worms were shorter-lived ($p=0.0007$, 0.003). It was previously noted that *rol-6* transformant strains show significant interstrain variation in life span (Don Riddle, University of British Columbia, personal communication, see Discussion).

5.2.2. Effect of backcrossing in the *sir-2.1* over-expresser strain LG100

LG100 was generated via transgene microinjection followed by transgene integration using gamma irradiation. Such radiation induces single strand breaks in the DNA to integrate the extrachromosomal transgenic DNA array into the genome (Riddle, 1997). This can also cause second site mutations, that may affect life span (Partridge and Gems, 2007). Therefore, to avoid unwanted background effects, it is standard procedure to extensively backcross transgenic animals to a wildtype background after radiation mediated transgene integration. A minimum of five rounds of backcrossing is

advisable. However, LG100 was not backcrossed prior to the reported experiments (Tissenbaum and Guarente, 2001); CGC strain record and WormBase).

To exclude the possibility that the observed increase in life span is caused by background effects (e.g. a second site mutation) rather than *sir-2.1* over-expression, Josh McElwee backcrossed LG100 five times to N2. The Rol phenotype was used to follow the *geIn3* transgene during the backcrossing. In a preliminary trial he saw no extension of life span in the out-crossed derivative of LG100, GA468 (data not shown). I then set out to verify this finding. Life span experiments were performed at 20˚C. To prevent progeny production, one day before the start of the life span experiments FUdR was applied to the plates to a final concentration of either 10 µM or, as in the original paper (Tissenbaum and Guarente, 2001), 40 µM (see Materials & Methods, section 2.2.4.).

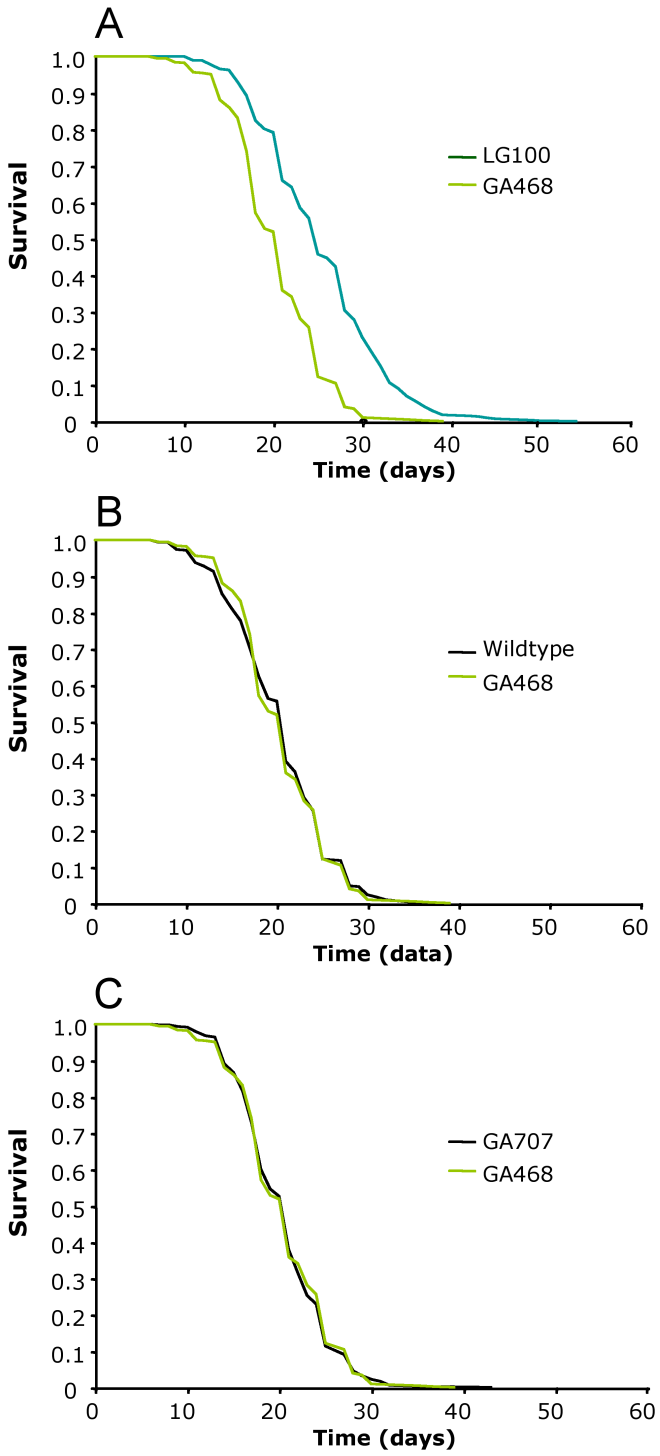


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Figure 5.2: Effect of out-crossing on life span in LG100. Genotypes: N2 wildtype, LG100 *geIn3 [sir-2.1 rol-6(su1006)]*, GA468 *geIn3 [sir-2.1 rol-6(su1006)]* and GA707 *wuEx166 [rol-6(su1006)].* **(A-C)** Life span curves of the out-crossed (5x) *sir-2.1* over-expresser GA468 compared to LG100, wildtype and *rol-6* control GA707 worms. Life span curves represent the combined data of six independent biological replicates. The statistical analysis is presented in Table 5.2.

Table 5.2: Effect of out-crossing on life span in LG100. The trials were performed at 20˚C, with FUdR added to 10 µM (trial 4-6) or 40 µM (trial 1-3) final concentration. Trial [wF] shows a trial without FUdR. Genotypes: N2 wildtype, GA707 *wuEx166 [rol-6(su1006)],* LG100 *geIn3 [sir-2.1 rol-6(su1006)]*, GA468 *geIn3 [sir-2.1 rol-6(su1006)]*. [C] combined data, *p*, log rank test. [n] Independent biological replicate number.

Strain	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* wildtype**	***p vs.* wildtype**	**% *vs.* GA707**	***p vs.* GA707**	**% *vs.* LG100**	***p vs.* or LG100**
Wildtype	**[C] 598/228**	**20.4**						
	[1] 84/43	18.9						
	[2] 79/48	19.9						
	[3] 62/63	22.6						
	[4] 86/34	19						
	[5] 164/9	21						
	[6] 123/31	20.1						
	[wF] 104/82	20.3						
GA707	**[C] 440/226**	**20.5**	**+0.5**	**0.66**				
	[1] 81/49	21	+11.1	0.01				
	[2] 96/29	21.8	+9.5	<.0001				
	[3] 75/50	23.6	+4.4	0.13				
	[4] 63/53	17	-10.5	0.0007				
	[5] 44/19	18.5	-11.9	0.003				
	[6] 81/79	19.2	-4.5	0.10				
LG100	**[C] 678/115**	**25.6**	**+25.5**	**<.0001**	**+24.9**	**<.0001**		
	[1] 91/17	26.6	+40.7	<.0001	+26.7	<.0001		
	[2] 119/7	31.8	+59.8	<.0001	+45.9	<.0001		
	[3] 116/6	26.3	+16.4	0.0001	+11.4	0.002		
	[4] 106/29	21.9	+15.3	<.0001	+28.8	<.0001		
	[5] 146/7	24.9	+18.6	<.0001	+35.6	<.0001		
	[6] 100/49	21.1	+5	0.02	+9.9	0.004		
GA468	**[C] 523/324**	**20.5**	**+0.5**	**0.90**	**0**	**0.82**	**-19.9**	**<.0001**
	[1] 77/48	18.8	-0.5	0.91	-10.5	0.0007	-29.3	<.0001
	[2] 71/64	21.7	+9	0.0007	-0.5	0.67	-31.8	<.0001
	[3] 66/59	21.1	-6.6	0.27	-10.6	0.002	-19.8	<.0001
	[4] 68/75	17.8	-6.3	0.01	+4.7	0.14	-18.7	<.0001
	[5] 167/9	21.5	+2.4	0.83	+16.2	0.0006	-13.7	<.0001
	[6] 74/69	19.7	-2	0.82	+2.6	0.28	-6.6	0.03
	[wF] 137/21	20	-1.5	0.49				

In four trials, GA468 showed no significant difference in life span to wildtype (trials [1], [3], [5] and [6], range: wildtype 18.9-22.6 days *vs.* GA468 17.8-21.7 days). Here, GA468 showed slight differences, such as a 9% increase to a 6.3% decrease in mean life span. In trial two, GA468 was significantly longer-lived by 9% than wildtype animals, whereas trial four showed a reduction in mean life span of 6.3%.

In three trials, GA468 did not show any statistical difference to *rol-6* controls (trials 1, 3 and 5; range: 17-23.6 days). Slight variations in mean life span, ranging from a 4.7% increase up to a 0.5% decrease could be detected. Twice GA468 was significantly shorter-lived than *rol-6* control animals by 10.6% and 10.5% respectively (trial [1] and [3]), whereas once a significant increase of 16.2% was observed. These variations were also caused by the previously mentioned interstrain variation in life span of *rol-6* lines. Overall, these results robustly confirm the above-mentioned rumours and Josh McElwee's preliminary finding that backcrossing of LG100 abolishes the increase in life span. To exclude the possibility that FUdR somehow inhibits the life span increasing effect of the transgene, I added one trial without FUdR ([wF]). Here the *sir-2.1* over-expressing worms showed also no increased life span (see [wF] 20.3 days *vs.* 20 days, p=0.49).

5.2.2.1. Out-crossing of LG100 does not affect *sir-2.1* over-expression

We next investigated the basis of the effect of out-crossing. One possibility is that this has somehow affected *geIn3* in such a way as to reduce transgene over-expression. Therefore the *sir-2.1* over-expression was tested by analysing the SIR-2.1 protein levels via western blotting. These protein measurements of both, GA468 and its not out-crossed predecessor LG100, were conducted by Dr Filipe Cabreiro (Burnett et al., 2011) (see Materials & Methods, section 2.2.13.).

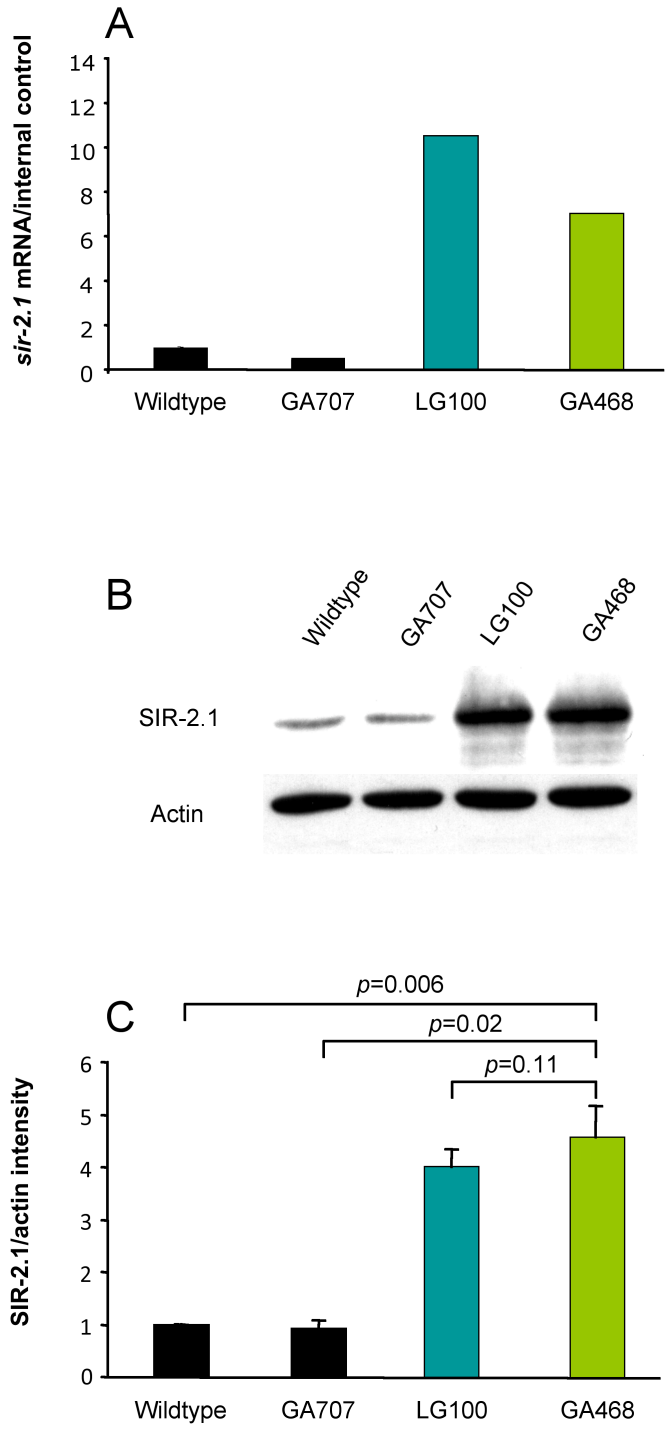


Figure 5.3: ***sir-2.1* mRNA and protein levels after backcrossing**. **(A)** Real-Time (RT) PCR showing *sir-2.1* mRNA levels of GA468 strain compared to its not out-crossed derivative LG100, wildtype and *rol-6* controls. *ama-1* was used as an internal control. This RT-PCR was carried out once, on one biological replicate, therefore I could not perform statistical analysis. Each column represents the mean of three technical replicates. **(B)** Shows a representative Western blot done by Dr Filipe Cabreiro (Burnett et al., 2011). The graph (**C)** shows the quantification of the SIR-2.1 protein levels relative to actin protein levels. Each column represents the mean of three biological replicates. The error bars show the S.E.M between the three technical replicates**.** Student's T-test was used for statistical analysis.

A preliminary Real-Time (RT) PCR tested the *sir-2.1* levels before and after backcrossing and revealed thus *sir-2.1* mRNA levels were still elevated in GA468. This was confirmed by Western blots performed by Dr Filipe Cabreiro. Here, the original *sir-2.1* over-expresser LG100 shows 256.1% higher SIR-2.1 levels than wildtype and 333.3% higher than *rol-6* animals. After the backcrossing, GA468 still has 289.3% higher SIR-2.1 levels than wildtype and 373.8% higher than *rol-6* controls. This shows that the out-crossing did not affect the *sir-2.1* over-expression. It also indicates that *sir-2.1* over-expression is not the reason for the extended life span of LG100.

5.2.3. Does a deleterious second site mutation suppress the effects of *sir-2.1* on life span in GA468

Another formal possibility is that backcrossing has introduced an unlinked allele that suppresses the effect of *sir-2.1* over-expression on longevity. To probe this, we asked whether the out-crossed derivative of LG100, GA468, is still competent for life span extension. We tested this by verifying that *daf-2* RNAi could still increase its life span. In most strains this causes an up to 50% increase in *C. elegans* life span. The out-crossed derivative of LG100, GA468 was kept for two generations on control and *daf-2* RNAi cultures prior the start of the life span measurements to guarantee an efficient reduction of *daf-2* expression levels. One day prior the start of life span measurements, FUdR was applied topically to the life span plates to a final concentration of 10 µM to prevent development of offspring (see Materials & Methods, section 2.2.4.).

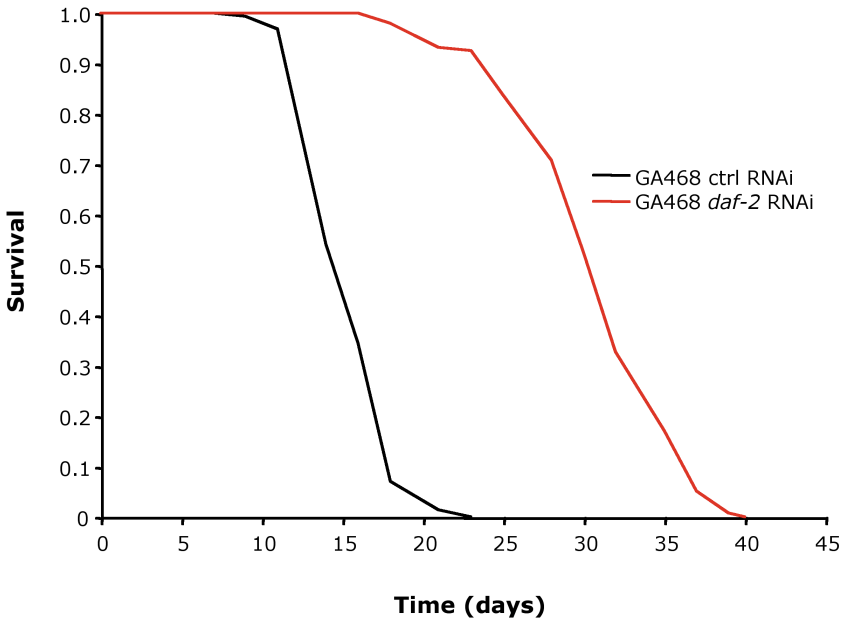


Figure 5.4: Effect of reduced *daf-2* expression on life span in GA468. Genotype: GA468 *geIn3 [sir-2.1 rol-6(su1006)].*

Table 5.4: Effect of reduced *daf-2* expression on life span in GA468. The trials were performed at 25˚C, with 10 µM FUdR. Genotypes: GA468 *geIn3 [sir-2.1 rol-6(su1006)]. p*, log rank test.

Strain	RNAi	Deaths/ censored	Mean life span (days)	% *vs.* ctrl	*p vs.* ctrl
GA468	ctrl	154/11	15.9		
GA468	*daf-2*	143/15	31	+95	<.0001

GA468 proved to be significantly longer-lived by 95% on *daf-2* RNAi than on control RNAi. Dr Filipe Cabreiro repeated this life span trial with the same result (data not shown). This argues against the possibility that an unlinked allele results in a loss of capacity for increased life span. However, this test does not exclude the possibility (though unlikely) that such an allele, present in the N2 cultivar used in our work, selectively suppresses the effect of *sir-2.1* over-expression on life span.

5.2.4. Effect of reduced *sir-2.1* expression levels in LG100

One possible explanation for the effect of backcrossing on the longevity of LG100 is that a second site mutation rather than *geIn3* is the cause of the extended life span. If this were the case, then knock down of *sir-2.1* expression should not suppress LG100 longevity. RNA mediated interference (RNAi) is a means to knock down expression of a specific gene (see Chapter 3 and Materials & Methods, section 2.2.4.). Therefore we knocked down *sir-2.1* expression by RNAi in the LG100 strain. Worms were kept for two generations on the RNAi cultures prior the start of the life span measurements to ensure an efficient reduction of *sir-2.1* expression levels, and transferred every day within the first week to avoid progeny contamination.

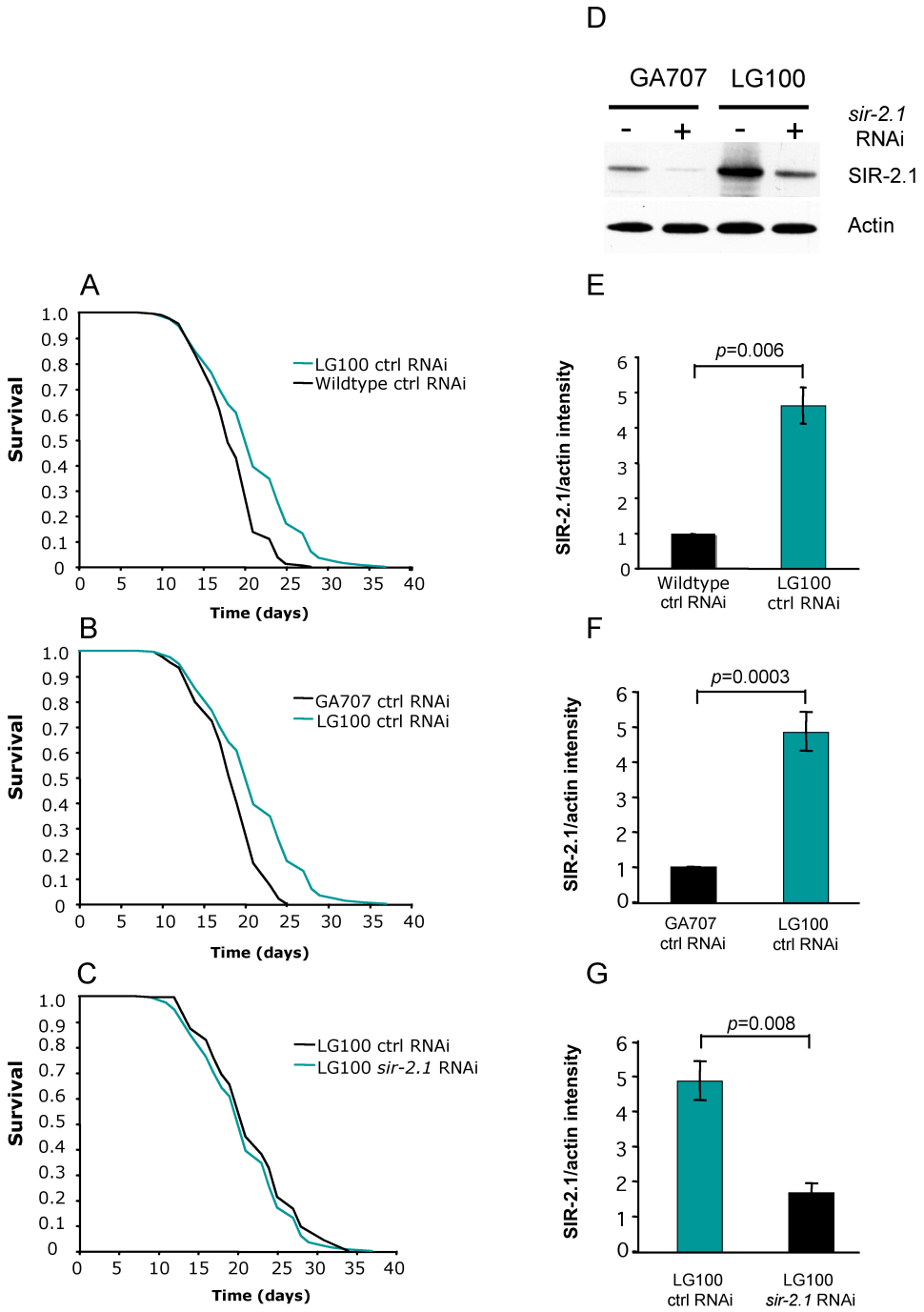


Figure 5.5: Effect of *sir-2.1* RNAi on life span in LG100. Genotypes: N2 wildtype, GA707 *wuEx166 [rol-6(su1006)],* LG100 *geIn3 [sir-2.1 rol-6(su1006)]*. **(A-B)** Life span curve of LG100 on control RNAi compared to wildtype animals and *rol-6* control animals. **(C)** Effect of *sir-2.1* RNAi on LG100. **(D)** A representative Western blot showing the SIR-2.1 levels in LG100 and the *rol-6* control on control and *sir-2.1* RNAi performed by Dr Filipe Cabreiro (Burnett et al., 2011). **(E-G)** The graphs show the quantification using actin. Each column represents the mean of three biological replicates and the Student's T-test was used for statistical analysis. The error bars show the S.E.M. Life span data represents the combined data of two independent biological replicates. The statistical analysis is presented in Table 5.4.

Table 5.4: Effect of *sir-2.1* RNAi on life span in LG100. The trials were performed at 20˚C, without FUdR. Genotypes: N2 wildtype, GA707 *wuEx166[rol-6 (su1006)],* LG100 *geIn3 [sir-2.1 rol-6(su1006)].* [C] combined data. Ctrl RNAi is the L4440 plasmid vector (negative control). *p,* log rank test. [n] Independent biological replicate number.

Strain	RNAi	Deaths/ censored	Mean life span (days)	% *vs.* wildtype ctrl	*p vs.* wildtype ctrl	% *vs.* GA707 ctrl	*p vs.* GA707 ctrl	% *vs.* LG100 ctrl	*p vs.* LG100 ctrl
Wildtype	Ctrl	**[C] 210/11**	**18.7**						
		[1] 118/9	17.9						
		[2] 92/2	19.7						
Wildtype	*sir-2.1*	**[C] 166/74**	**18.4**	**-1.6**	**0.44**	**-0.5**	**0.52**		
		[1] 117/8	18.2	+1.7	0.45	-1.1	0.22		
		[2] 49/66	19.1	– 3	0.36	+3.2	0.33		
GA707	Ctrl	**[C] 194/47**	**18.5**	**-1.1**	**0.50**				
		[1] 97/28	18.4	+2.8	0.07				
		[2] 97/19	18.5	-6	0.02				
GA707	*sir-2.1*	**[C] 177/71**	**19.4**	**+3.7**	**0.02**	**+4.9**	**0.006**		
		[1] 107/16	19.1	+6.7	0.0004	+3.8	0.04		
		[2] 68/55	19.4	-1.5	0.77	+4.9	0.08		
LG100	Ctrl	**[C] 222/25**	**20.9**	**+11.8**	**<.0001**	**+13**	**<.0001**		
		[1] 117/9	19.8	+10.6	<.0001	+7.6	0.01		
		[2] 105/16	22.3	+13.2	<.0001	+20.5	<.0001		
LG100	*sir-2.1*	**[C] 207/44**	**21.9**	**+17.1**	**<.0001**	**+18.4**	**<.0001**	**+4.8**	**0.06**
		[1] 108/17	21.1	+17.9	<.0001	+14.7	<.0001	+6.6	0.05
		[2] 99/27	22.7	+15.2	<.0001	+22.7	<.0001	+1.8	0.29

LG100 was repeatedly long-lived compared to wildtype (17.9, 19.7 days *vs.* 19.8, 22.3 days, $p<.0001$) and *rol-6* controls (18.4, 18.5 days, $p<.0001$) on control RNAi. Here, the increase in mean life span was 11.8% (10.8% and 13.2%) compared to wildtype, and 13% (7.6% and 20.5%) compared to *rol-6* animals. The observed interstrain variations in the *rol-6* control was again seen. The variation in mean life span compared to wildtype ranged from a 2.8% increase up to a 6% decrease. However, the combined data indicated no significant difference between wildtype and *rol-6* strains ($p=0.50$).

LG100 did not show any difference in life span on control RNAi compared to *sir-2.1* RNAi (19.8, 22.3 days *vs.* 21.1, 22.7 days, respectively). Western blot analysis, performed by Dr Filipe Cabreiro, confirmed that *sir-2.1* RNAi lowered the SIR-2.1 protein levels by 65.8% compared to control RNAi ($p=0.008$). On control RNAi, the SIR-2.1 levels were 388% higher than *rol-6* animals ($p=0.0003$) and 377% higher than wildtype ($p=0.006$).

Notably, the life span on *sir-2.1* RNAi was increased by 4.8% compared to control animals, but the difference did not quite reach statistical significance ($p=0.06$). Therefore *sir-2.1* RNAi did not suppress the extended life span of LG100, supporting the conclusion from the out-crossing tests that the increased life span of the LG100 strain is not *sir-2.1* dependent. This pattern of increased survival on *sir-2.1* RNAi was also observed in the *rol-6* control. Here, life span was increased by 4.9% compared to control animals, and this time the difference was significant ($p=0.006$).

Comparing the RNAi data with the data on OP50 bacteria, it also becomes evident that the LG100 strain is not as long-lived on the RNAi

bacteria HT115 as on OP50 (mean of 25.3 days on OP50 *vs.* 20.9 days on HT115). This is consistent with two studies reporting mean life spans of 19.5±1.1 days (Morselli, 2010) and 21.2±0.6 days for LG100 on HT115 bacteria (Wang et al., 2006). *E. coli* strain-dependent effects on *C. elegans* life span are not uncommon (Maier et al., 2010). These results imply that whatever it is that increases life span, its effects are sensitive to the *E. coli* strain used as a food source.

5.2.5. A dye-filling defect (Dyf) mutation causes the life span extension in LG100

From communication with L. Guarente, we learned that S. S. Lee (Cornell University, USA) had discovered that LG100 has a dye-filling defective (Dyf) phenotype. Dyf mutants exhibit defects in sensory neurons resulting in absence of fluorescent dye uptake into neurons (see Introduction, section 1.4.8.). Furthermore, she noted that the extended life span co-segregated with the *dyf* mutation, not with the *geIn3* transgene (S. S. Lee, personal communication). This suggests the presence in LG100 of a *dyf* mutation unlinked to *geIn3*. We named this allele *dyf-?(wu250)*. The Cornell group did not wish to pursue these observations any further, but encouraged us to replicate them. I therefore performed a dye-filling test (see Materials & Methods, section 2.2.8.) with LG100 out-crossed derivative GA468, and confirmed the former but not the later is Dyf (see Figure 5.6). This supported the view that a second site *dyf* mutation is the reason for the extension in life span of LG100, not *sir-2.1* over-expression.

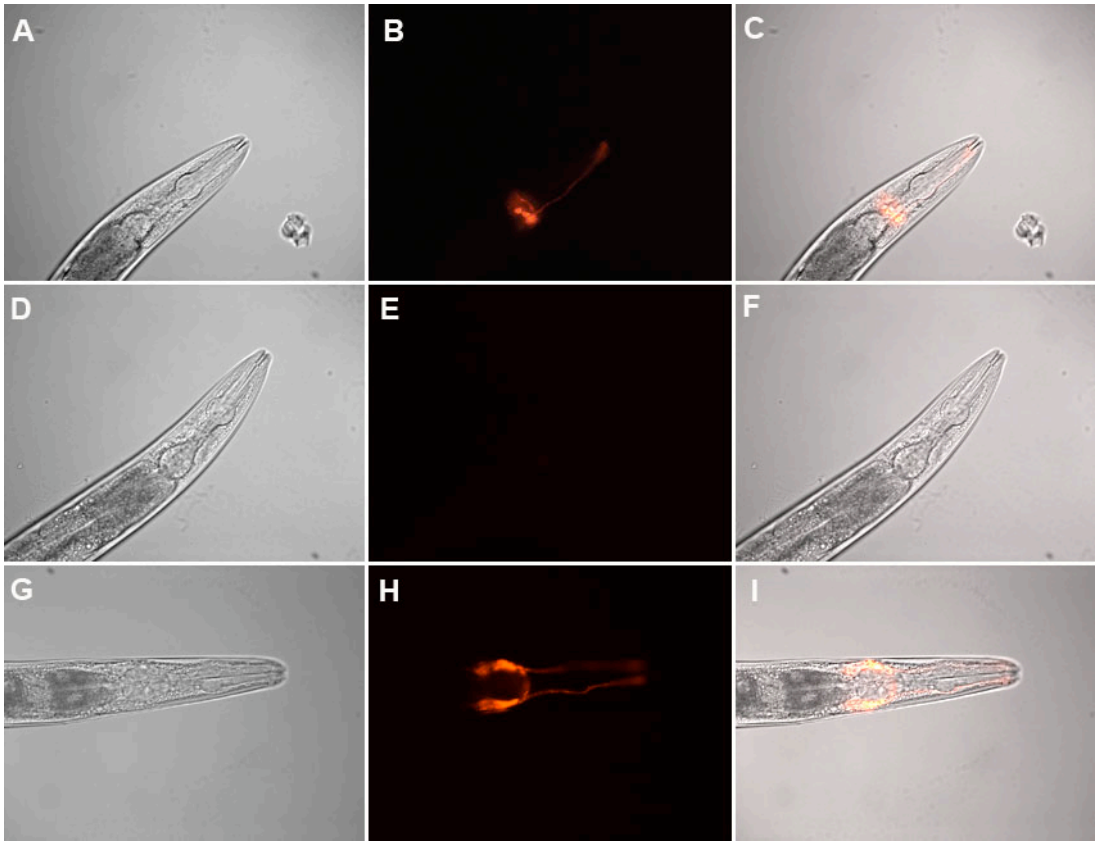


Figure 5.6: LG100 but not GA468 has a dye-filling (Dyf) defect. The first column of pictures **(A, D and G)** shows bright field images, the second column **(B, E and H)** epifluorescence images and the third one **(C, F and I)** the overlay of both. The wildtype **(A-C)** and GA468 **(G-I)** show a normal staining of the amphidial neurons using the carbocyanin dye DiI (1,1'-dioctadecyl-3,3,3', 3'-tetramethylindo-carbocyanine perchlorate), whereas the LG100 **(D-F)** does not.

To test this further, I segregated the *dyf-?(wu250)* mutation away from the *geIn3* transgene in order to test this effect on life span. During the segregation I used the Dyf phenotype to follow the *dyf-?(wu250)* mutation, and the Rol phenotype to follow the *geIn3* transgene. The segregation was performed by crossing LG100 with N2 males, selfing the F_1 generation and then screening the F_2 generation for Dyf, non-Rol, and Rol, non-Dyf individuals (see Figure 5.6). F_3 progeny of Rol, non-Dyf F_2s were checked to identify those that were homozygous for *geIn3* and the wildtype allele of *dyf-?* To verify the segregation, Dr Filipe Cabreiro analysed again the SIR-2.1 protein levels of the independent lines via Western blotting.

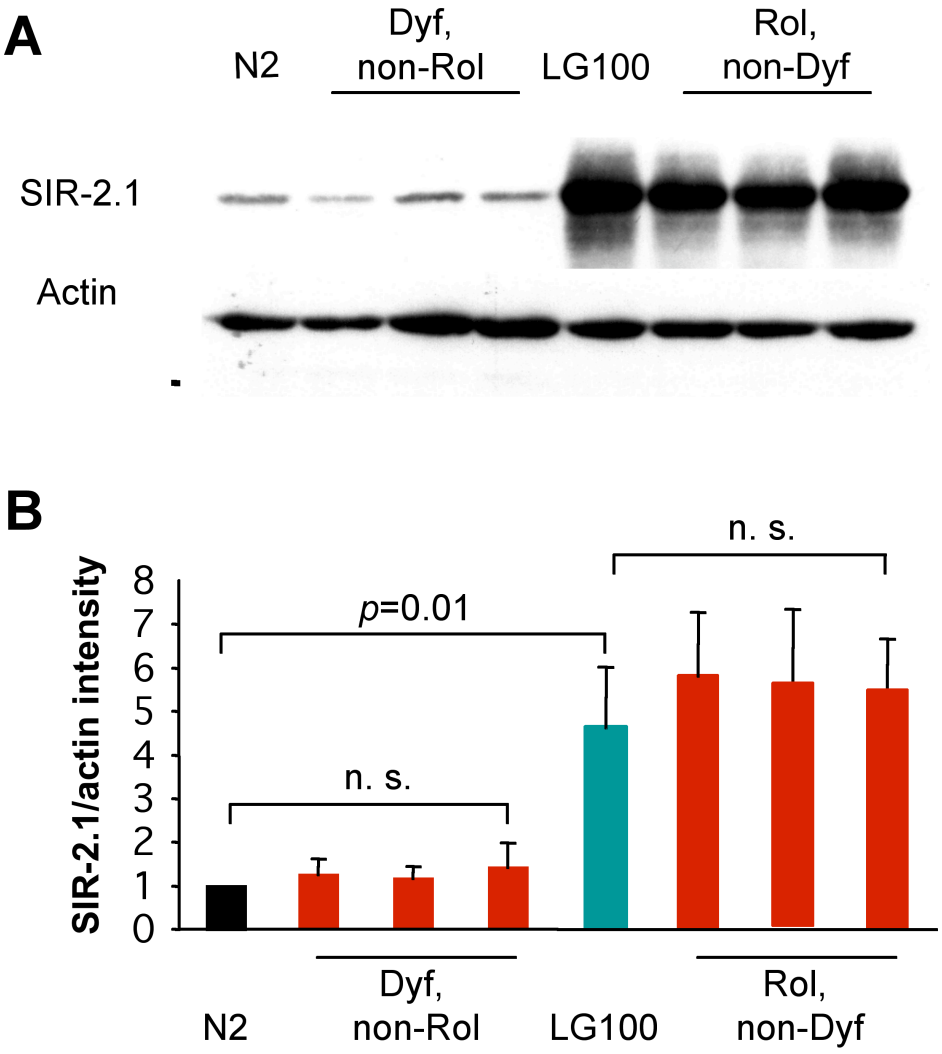


Figure 5.7: SIR-2.1 levels of Dyf, non-Rol and Rol, non-Dyf lines. (A) Shows a representative western blot done by Dr Filipe Cabreiro (Burnett et al., 2011). **(B)** The graph shows the quantification of SIR-2.1 levels using actin. Each column represents the mean of three biological replicates. The error bars show the S.E.M. The Student's T-test was used for statistical analysis.

Three independent Dyf, non-Rol lines and three independent Rol, non-Dyf lines were isolated and their life spans tested. Combined data of the Dyf, non-Rol and Rol, non Dyf lines of two biological trials showed that the worms carrying the Dyf mutation were significantly longer-lived than the Rol segregants (p<.0001), but they were also significantly shorter-lived than LG100 (p<.0001) (data not shown). However, the wildtype N2 strain used for the segregation and life span measurements was repeatedly short-lived in both trials: mean life span of 15.5 and 15.7 days under plate culture conditions at 20˚C. N2 hermaphrodites should show a mean life span of approximately 18-20 days at the temperature used (Gems and Riddle, 2000). The short life span of N2 in this case strongly implies that this is a short-lived mutant derivative of N2. Occurrence of such short-lived derivatives is a recurrent pitfall in *C. elegans* ageing studies (Gems and Riddle, 2000). This raises the concern that genetic determinants of short life span in the used N2 line had been inherited by Rol, non-Dyf and Dyf, non-Rol segregants. Therefore I repeated the construction of Rol, non-Dyf and Dyf, non-Rol lines using a N2 tested for its normal life span and more recently obtained from the CGC. After again preparing the six lines, I re-confirmed the dye-filling pattern of the segregants (Figure 5.8).

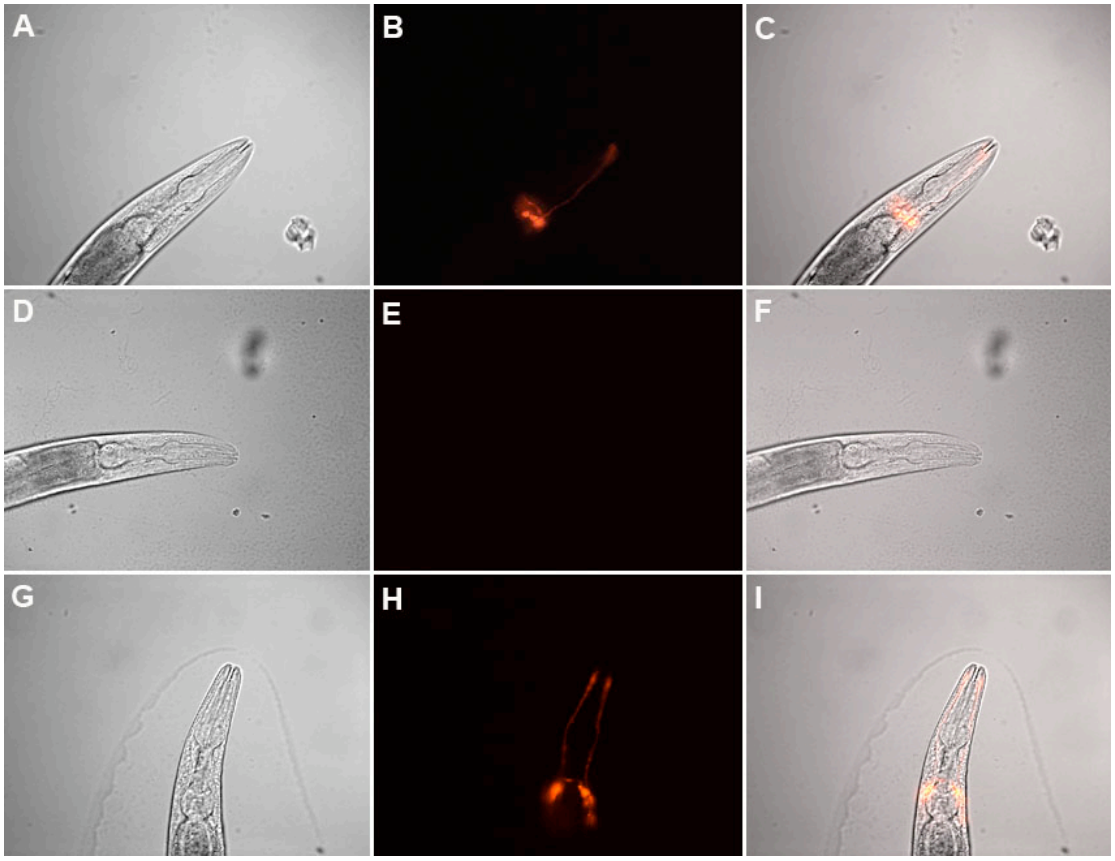


Figure 5.8: Dyf, non-Rol lines but not Rol, non-Dyf lines have a dye-filling (Dyf) defect. The first column of pictures **(A, D and G)** shows bright field images, the second column **(B, E and H)** epifluorescence images and the third one **(C, F and I)** the overlay of both. **A-C**, normal amphidial staining DiI in *C. elegans* wildtype animals. **(D-F)** Dyf, non-Rol segregant (GA918), without any detectable staining, a characteristic of the Dyf phenotype. **G-I**, Rol, non-Dyf segregant (GA920) with a normal pattern of dye-filling in the sensory neurons.

After the phenotypic confirmation of the segregants I tested the life span of the independent lines. Life span experiments were performed at 20˚C. One day before the start of the life span experiments FUdR was applied to the plates to a final concentration of 10 μM to avoid progeny contamination (see Materials & Methods, section 2.2.4.).

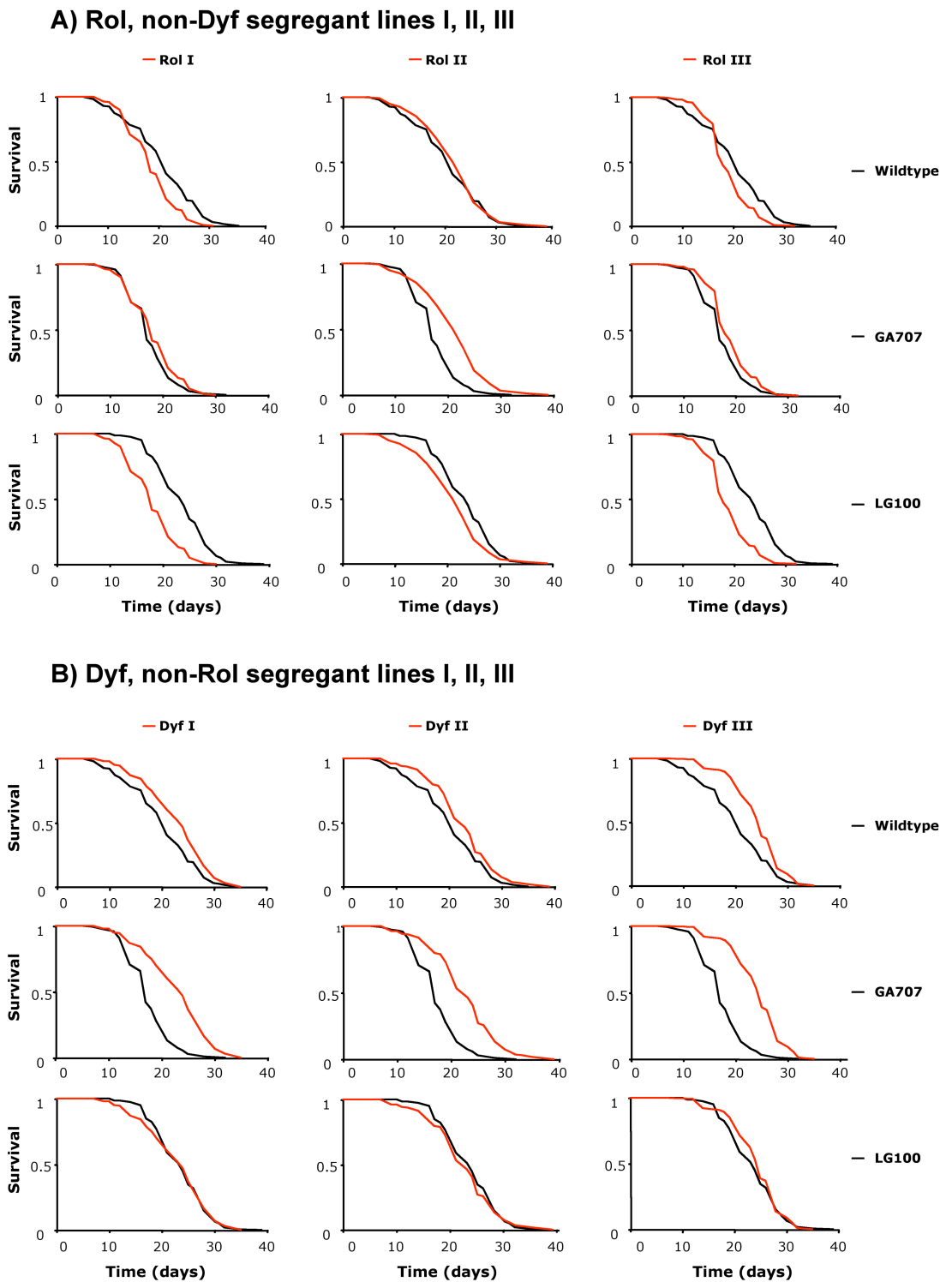


Figure 5.9: Longevity of *C. elegans* LG100 strain segregates with *dyf-?(wu250)* mutation. The trials were performed at 20˚C, with 10 µM FUdR. **A)** Shows the life span curves of the three independent Rol, non-Dyf lines compared to the wildtype, *rol-6* and LG100 animals. **B)** Shows the live span curves of the three independent Dyf, non-Rol lines compared to wildtype, *rol-6* and LG100 animals. The life span curves represent the combined data of two independent biological replicates. The statistical analysis is presented in Table 5.5.

Table 5.5: Longevity of *C. elegans* LG100 strain segregates with *dyf-?(wu250)* mutation .The trials were performed at 20˚C, with 10 µM FUdR. [C] combined data. [n] Independent biological replicate number. *p*, log rank test.

Strains	**Deaths/ censored**	**Mean life span**	**% *vs.* wildtype**	***p vs.* wildtype**	**% *vs.* GA707**	***p vs.* GA707**	**% *vs.* LG100**	***p vs.* LG100**
Wildtype	**[C] 250/43**	**20.4**						
	[1] 86/34	19						
	[2] 164/9	21						
GA707	**[C] 107/72**	**17.7**	**-13.2**	**<.0001**				
	[1] 63/53	17	-10.5	0.0007				
	[2] 44/19	18.5	-11.9	0.003				
LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*	**[C] 252/36**	**23.7**	**+16.2**	**<.0001**	**+33.9**	**<.0001**		
	[1] 106/29	21.9	+15.3	<.0001	+28.8	<.0001		
	[2] 146/7	24.9	+18.6	<.0001	+34.6	<.0001		
Rol, non-Dyf lines	**[C] 560/174**	**19.4**	**-4.9**	**0.0006**	**+9.6**	**<.0001**	**-18.1**	**<.0001**
I GA919 *geIn3 [sir-2.1 rol-6(su1006)]*	**[C] 207/87**	**18.3**	**-10.3**	**<.0001**	**+3.4**	**0.09**	**-22.8**	**<.0001**
	[1] 54/75	16.1	-15.3	<.0001	-5.3	0.11	-26.5	<.0001
	[2] 153/12	19	-9.5	<.0001	+2.7	0.76	-23.7	<.0001
II GA920 *geIn3 [sir-2.1 rol-6(su1006)]*	[2] 125/21	21.6	+2.9	0.81	+16.8	0.001	-13.3	<.0001
III GA921 *geIn3 [sir-2.1 rol-6(su1006)]*	**[C] 228/66**	**19.2**	**-5.9**	**<.0001**	**+8.5**	**0.002**	**-19**	**<.0001**
	[1] 95/55	17.4	-8.4	0.0004	+2.4	0.75	-20.5	<.0001
	[2] 133/11	20.4	-2.9	0.003	+10.3	0.06	-18.1	<.0001
Dyf, non-Rol lines	**[C] 647/256**	**23.5**	**+15.2**	**<.0001**	**+32.8**	**<.0001**	**-0.8**	**0.79**
I GA916 *dyf-?(wu250)*	**[C] 204/91**	**23.2**	**+13.7**	**<.0001**	**+31.1**	**<.0001**	**-2.1**	**0.81**
	[1] 55/80	20.5	+7.9	0.05	+20.6	<.0001	-6.4	0.07
	[2] 149/11	24.1	+14.8	0.0001	+30.3	<.0001	-3.2	0.91
II GA917 *dyf-?(wu250)*	**[C] 238/57**	**22.8**	**+11.8**	**0.0001**	**+28.1**	**<.0001**	**-3.8**	**0.36**
	[1] 107/46	21	+10.5	0.005	+23.5	<.0001	-4.1	0.12
	[2] 131/11	24.1	+14.8	0.0001	+30.3	<.0001	-3.2	0.93
III GA918 *dyf-?(wu250)*	**[C] 205/108**	**24.4**	**+19.6**	**<.0001**	**+37.9**	**<.0001**	**+3**	**0.19**
	[1] 77/86	23	+21.1	<.0001	+35.3	<.0001	+5	0.10
	[2] 128/22	25.3	+20.5	<.0001	+36.8	<.0001	+1.6	0.84

The Rol, non-Dyf line Rol I and III are 10.3% and 5.9% shorter-lived than wildtype (16.1, 19 days and 17.4, 20.4 days *vs.* 19, 21.1 days respectively, $p<.0001$). The Rol line II shows a 2.9% increase in life span compared to wildtype, but this is not significant ($p=0.81$).

Comparing the Rol, non-Dyf lines to the *rol-6* control, Rol I showed no significant difference (16.1, 19 days *vs.* 17, 18.5 days respectively, $p=0.09$), whereas Rol lines II and III showed a 16.8% and 8.5% increase in life span (21.6 days and 17.4, 20.4 days, $p=0.001$ and $p=0.001$ respectively).

All Dyf, non-Rol lines are longer-lived than wildtype (range: 7.5-20.7% increase in mean life span, in most cases statistical significance reached $p<.0001$) and *rol-6* animals (range: 19.9-38.2% increase in mean life span, in all cases statistical significance reached $p<.0001$). No significant difference in life span relative to LG100 could be detected in two biological replicates within the three independent Dyf, non-Rol lines (combined range: 20.5-25.3 days *vs.* 21.9, 24.9 days respectively, $p=0.79$).

However, the wildtype and *rol-6* life spans are at the higher and lower ends, respectively, of the expected 18-20 day range of wildtype mean life span. Since the mean life span of each single Rol, non-Dyf line lies within normal parameters for wildtype *C. elegans* worms the segregants are not considered to be short-lived.

Overall, the life span measurements reconfirmed that the longevity of LG100 segregates with the Dyf mutation and not the *sir-2.1* transgene. This demonstrates that *sir-2.1* over-expression is not the cause of the extended life span in this strain.

5.2.6. Mapping the *dyf-?* locus

5.2.6.1. Two point mapping

To characterize *dyf-?(wu250)* further, I began to map the mutation. *C. elegans* has five autosomes (I-V) and one X chromosome. Hermaphrodites are diploid for all six chromosomes, whereas males are haploid for the X chromosome. To identify the chromosome in which a given mutation is located, a variety of phenotypic markers can be used. The genetic distance between two linked genes can be estimated thanks to the occurrence of meiotic recombination. The closer two genes are to each other, the less frequently recombination occurs. One map unit is defined as a 1% recombination frequency. The first step to map a mutation is two-point mapping. This mapping step is used to determine the specific chromosome the mutation is located (see Materials & Methods, section 2.2.11.4.1.). For my two-point mapping I used the mapping strain DA438 generated by L. Avery. This strain has one or more phenotypic marker on each chromosome, as follows: *bli-4(e937) I, rol-6(e187) II, daf-2(e1368) vab-7(e1562) III, unc-31(e928)IV, dpy-11(e224) V, lon-2(e678) X* (Maduro and Pilgrim, 1995) see Introduction, section 1.4.3. for description of phenotypic markers). I obtained it from the CGC.

Using this strain, it is possible to identify the chromosome carrying a given mutation (here *dyf-?*) with a single cross. The *dyf-?* and DA438 mapping strain were crossed, and Dyf animals picked from the F_2. The phenotypic marker that occurs at the lowest frequency in the F_3 is deduced to be on the same chromosome as the *dyf* mutation. 40 Dyf F_2s were picked and their offspring screened for the phenotypic markers (Figure 5.10).

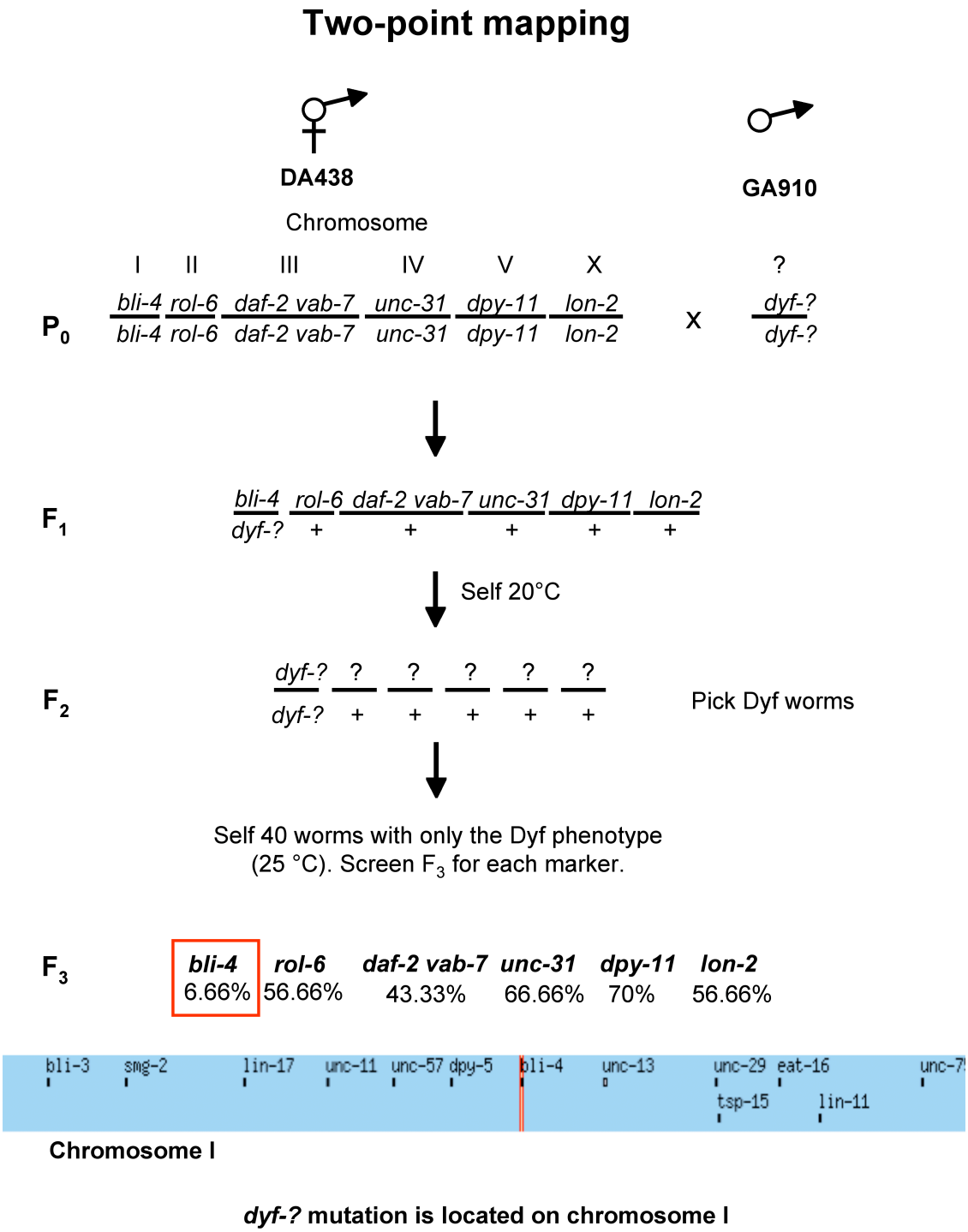


Figure 5.10: Two point mapping of the Dyf-? mutation. The mapping strain DA438 *bli-4(e937) I, rol-6(e187) II, daf-2(e1368) vab-7(e1562) III, unc-31(e928) IV, dpy-11(e224) V, lon-2(e678) X* was crossed into the Dyf segregant GA910 *dyf-?(wu250)* (P_0), the F_1 generation was selfed at 20°C, 40 worms with the Dyf phenotype only were picked in the F_2 generation and selfed at 25°C. This step was performed at 25°C because the *daf-2 vab-5* marker is temperature sensitive. The low frequency of Bli segregants in the F_3 generation indicated that the Dyf mutation is located on chromosome I. The chromosome map was taken from WormBase.

Of these, the *bli-4* phenotype was rarest (6.66%). This indicates that the *dyf* mutation is on chromosome I. The *bli-4* gene is located in the centre of chromosome I. I repeated the mapping a second time with the same result (data not shown).

5.2.6.2. Three-point mapping

The next step after two-point mapping is three-point mapping. This step narrows down the area on the chromosome containing the mutation of interest (see Materials & Methods, section 2.2.11.4.2.). I chose the mapping strain DR435 carrying *dpy-5(e61) unc-13(e51) I* for further mapping and obtained it from the CGC. These two mutations are close to the right and left side of the *bli-4* locus.

The phenotypic marker appearing the least frequent in the cross-progeny indicates if the Dyf mutation is located on the right (in case of higher % of *dpy-5* cross-progeny) or on the left (in case of higher % of *unc-13* cross-progeny). I started the mapping by crossing Dyf males (generated via heat-shock) into DA435 hermaphrodites (P_0) and selfed the F_1 generation. I continued from here in two different ways.

I picked as many Dpy, non-Unc (n=11) and Unc, non-Dpy (n=1) worms as I could find, selfed them and checked in the progeny for Dyf recombinants (Figure 5.11).

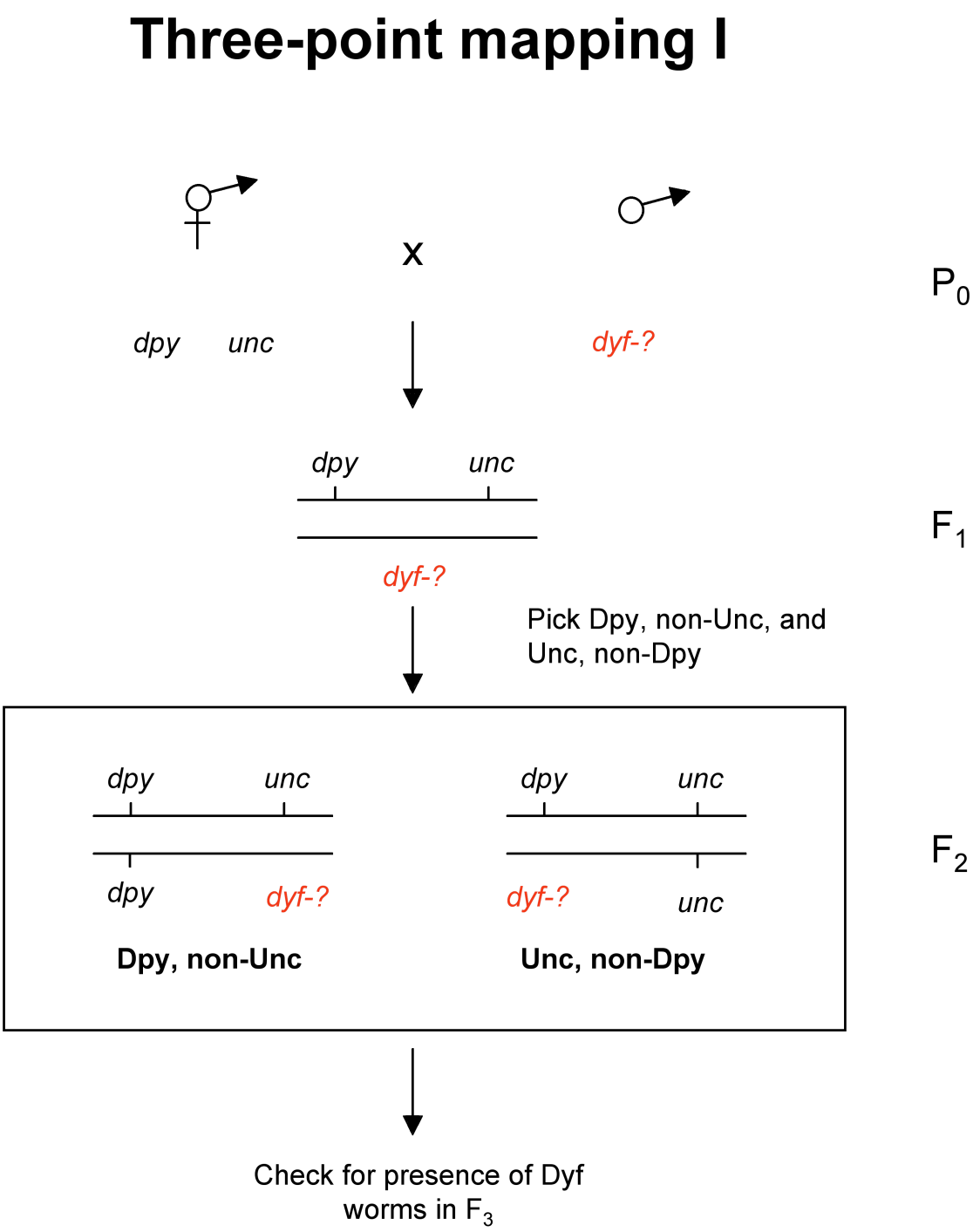
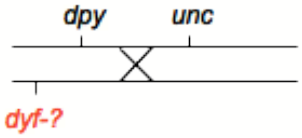
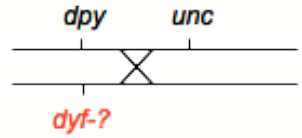
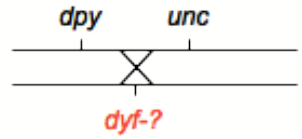
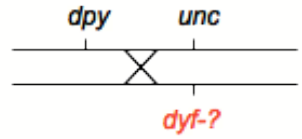
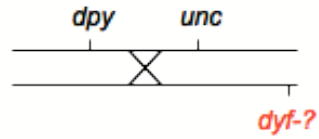


Figure 5.11: Three-point mapping of the Dyf-? mutation I. I crossed the mapping strain DR435 *dpy-5(e61) unc-13(e51)I* with the Dyf segregant GA910 *dyf-?(wu250)I* (P_0), and selfed the heterozygote F_1 generation. In the F_2 generation. I picked Dpy, non-Unc and Unc, non-Dpy worms, selfed them and the F_3 generation was screened for Dyf recombinants.

Possible locations of *dyf-?*

Position	Recombination	Predicted presence of Dyf		Observed
		Dpy, non-Unc	Unc, non-Dpy	
Left of *dpy-5*		No	Yes	X
Close to *dpy-5*		No	Yes	X
Between *dpy-5* and *unc-13*		Yes	Yes	✔
Close to *unc-13*		Yes	No	✔
Right of *unc-13*		Yes	No	✔

Mapping results in F_3	Dyf progeny
Dpy, non-Unc progeny	11/11
Unc, non-Dpy progeny	1/1
Dpy Unc Dyf progeny	6/11

Figure 5.12: Possible locations of *dyf-?(wu250)* and corresponding predicted phenotypes I. Mapping results show more Dpy Dyf than Unc Dyf recombinants.

Unexpectedly, 6/11 Dpy, non-Unc F_2 segregated Dpy Unc Dyf progeny. This implies that in these 6 worms, the chromosome with the parental genotype, *dpy-5 unc-13*, is actually recombinant and bearing *dyf-?(wu250)*. The only way this could happen is if the Dyf locus was at the far right of LGI. The distance between *unc-13* and the right end of LGI is approximately 25 map units.

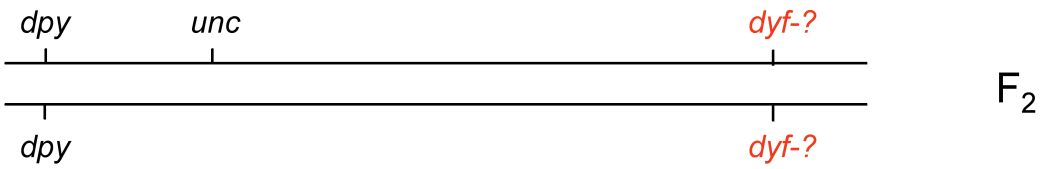


Figure 5.13: Probable location of *dyf-?(wu250).*

Both chromosomes are recombinant. For this to happen, *dyf* would have to be very far to the right of *unc-13*. By chance alone, ~50% of "parental chromosomes" are recombinant!

In the second way of locating the *dyf-?(wu250)*, I picked phenotypic wildtype worms with dye-filling defect (n=40), selfed them and screened the offspring for the phenotypic Unc or Dpy markers (Figure 5.14).

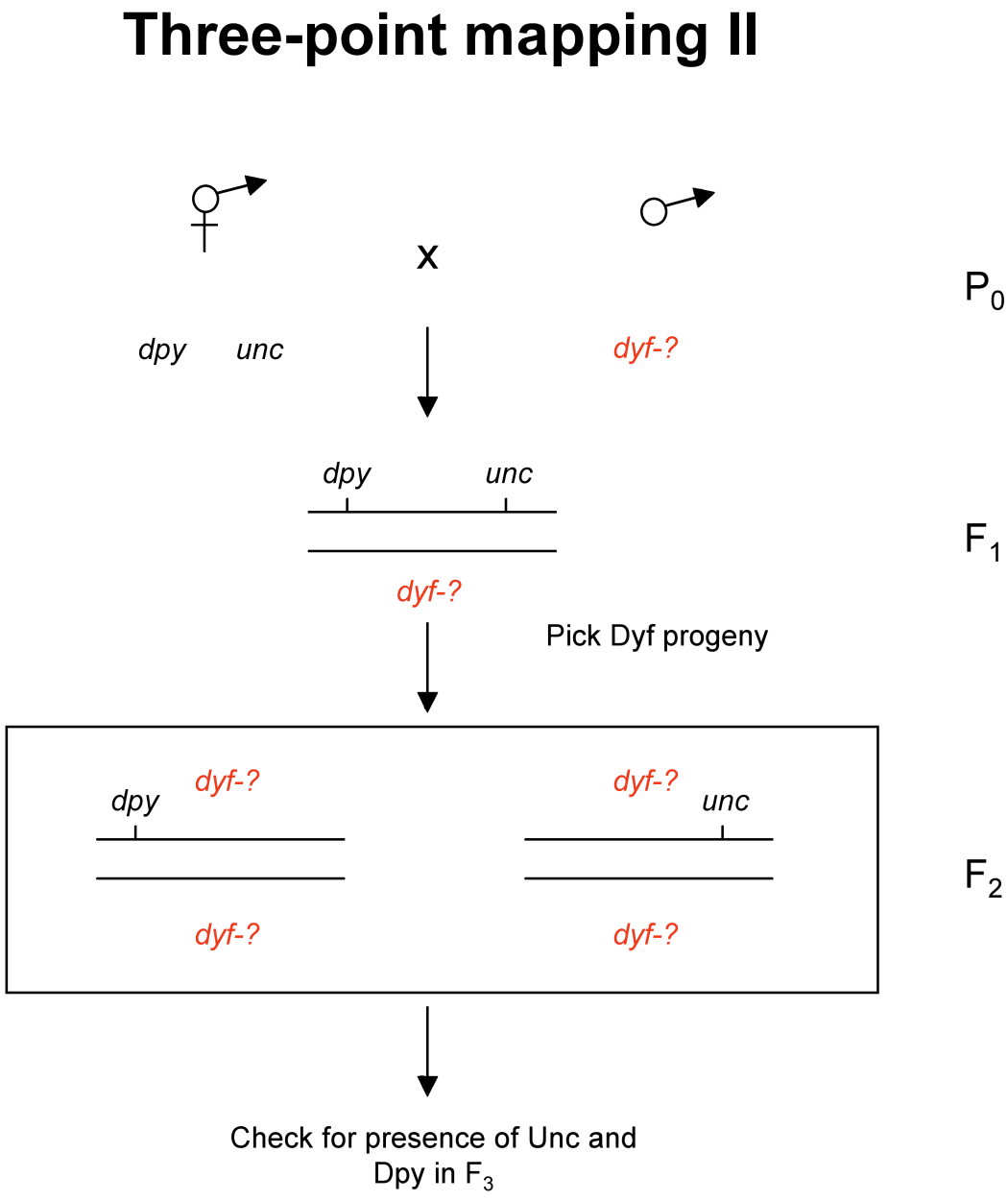
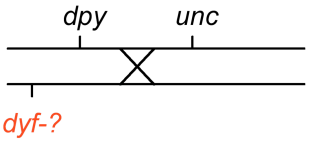
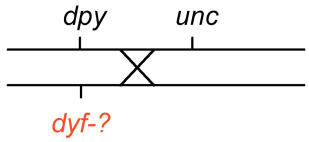
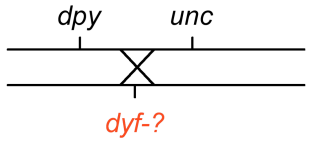
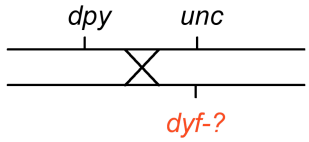
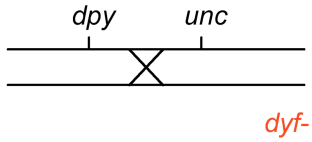


Figure 5.14: Three-point mapping of the Dyf-? mutation II. I crossed the mapping strain DR435 *dpy-5(e61) unc-13(e51)I* with the Dyf segregant GA910 *dyf-?(wu250)I* (P_0), and selfed the heterozygote F_1 generation. In the F_2 generation I picked Dyf worms, selfed them and the F_3 generation was screened for Unc and Dpy recombinants.

Dyf segregants

Position	Recombination	Predicted presence of Dyf: Dpy	Unc	Dpy Unc
Left of *dpy-5*		(✔) Double recomb.	✔	✔
Close to *dpy-5*		X	✔	X
Between *dpy-5* and *unc-13*		(✔) Double recomb.	✔	✔
Close to *unc-13*		✔	X	X
Right of *unc-13*		✔	(✔) Double recomb.	✔

Mapping results in F_3	**Dyf progeny**
Dpy Dyf	8/40
Unc Dyf	3/40
Dpy Unc Dyf	10/40

Implies that the Dyf mutation is on the right side of *unc-13*

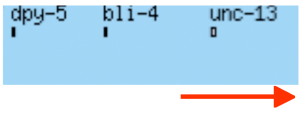


Figure 5.15: Possible locations of *dyf-?(wu250)* and corresponding predicted phenotypes II. The mapping result with this method showed a higher occurrence of Dpy Dyf and Dpy Unc Dyf recombinants.

The only model that fits the data is *dyf-?(wu250*) to the right of *unc-13*. The ratio of Dyf F_2 worms segregating Dpy *vs.* Unc Dpy F_3 worms supports the view that *dyf-?* is some distance to the right of *unc-13*, further than the 2.08 map units between *dpy-5* and *unc-13* (Figure 5.13).

5.2.6.3. Testing *dyf-1(nm335)* for *dyf-?(wu250)* identity

Preliminary three-point mapping data first suggested that the *dyf-?(wu250)* mutation is located to the left side of *bli-4* (data not shown). Not far from, the *bli-4* gene, 1.42 map units to its left side is the gene *dyf-1* mutation of which causes a similar dye-filling defect phenotype to that of *dyf-?.*

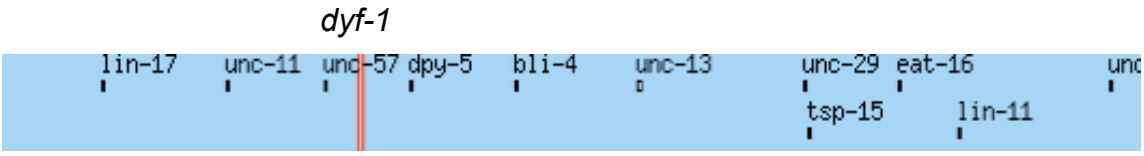


Figure 5.16: Map position of *dyf-1(mn335)*. The chromosome section was taken from WormBase.

At the time that the 2001 study was conducted, one allele of *dyf-1*, *mn335*, was available. To exclude the possibility that *dyf-?(wu250)* was this allele, I sequenced the region of the *dyf-1(mn335)* lesion in the Dyf segregant GA910. This showed that GA910 does not contain the 4 base pair insertion of the *nm335* allele:

...cccagcttcttctcaggcggttctagtg **----** cttgacgacgcgggaaattccgaattca... -- Wildtype
...cccagcttcttctcaggcggttctagtg TACA cttgacgacgcgggaaattccgaattca... -- *mn335*

5.2.7. Verifying the effect on life span of the duplication *mDp4*

The strain DR1786 carries the duplication *mDp4,* which includes the *sir-2.1* locus. The longevity of this strain was the original basis for the view that *sir-2.1* over-expression extends *C. elegans* life span (Tissenbaum and Guarente, 2001). Free duplications are usually generated by mutagenesis with ionizing radiation or UV light. In the case of *mDp4* gamma irradiation was used. *mDp4* is a large free duplication of chromosome IV of approximately 9.1 megabases in size.

The genotype of DR1786 is complex. It is described by the CGC as *dpy-13(e184) unc-24(e138); mDp4 [unc-17(e245)] (IV;?).* The duplication includes *dpy-13* and *unc-24* wildtype copies to rescue the mutant phenotype. The *dpy-13(e184)* allele is an in-frame deletion and is semi dominant. Therefore worms are strongly dumpy in homozygous state, whereas heterozygotes are weakly dumpy. *unc-24(e138)* animals are weak kinkers, which tend to back and often stay still in the shape of an omega. *unc-17(e2245)* result in severe coilers and a rather small and thin body morphology (Riddle, 1997).

During subculturing of DR1786, we saw not only Dpy Unc segregants (CGC strain description), but also other mutant phenotypes, such as Muv. This phenotype shows multiple vulva protrusions in adult hermaphrodites. Furthermore, I saw very slow moving, almost paralyzed worms that tended to curl, indicating the presence of another Unc mutation. After several generations of subculturing, I also recognized the frequent appearance of worms showing the Lon phenotype. These animals tend to be exceptionally

long compared to wildtype worms. These observed mutant phenotypes manifested mainly in adult animals and were hard to detect in larvae.

In our hands, the duplication strain did not show a typical N2 wildtype phenotype, but semi-dumpy one. The semi-dumpy phenotype is understandable, considering that *dpy-13(e184)* is a semi-dominant allele that causes a strong dumpy phenotype in homozygote state and a medium dumpy in heterozygotes.

The 2001 screen implicating *sir-2.1* as a gene promoting longevity included 35 different duplications. All were tested for effects on life span at 20°C, and four showed a significantly longer life span than wildtype animals in the same trial. The four strains and their corresponding duplications were DR1786 *mDp4* (F, mean of 19.4±0.6 days, n=50), KR1732 *hDp34* (C, mean of 17.3±0.5 days, n=36), RW6011 *mnDp34* (D, mean of 17.6±1.0 days, n=27) and SP125 *mnDp5* (M, mean of 18.8±1.0 days, n=31) (see Figure 5.17 for chromosomal locations).

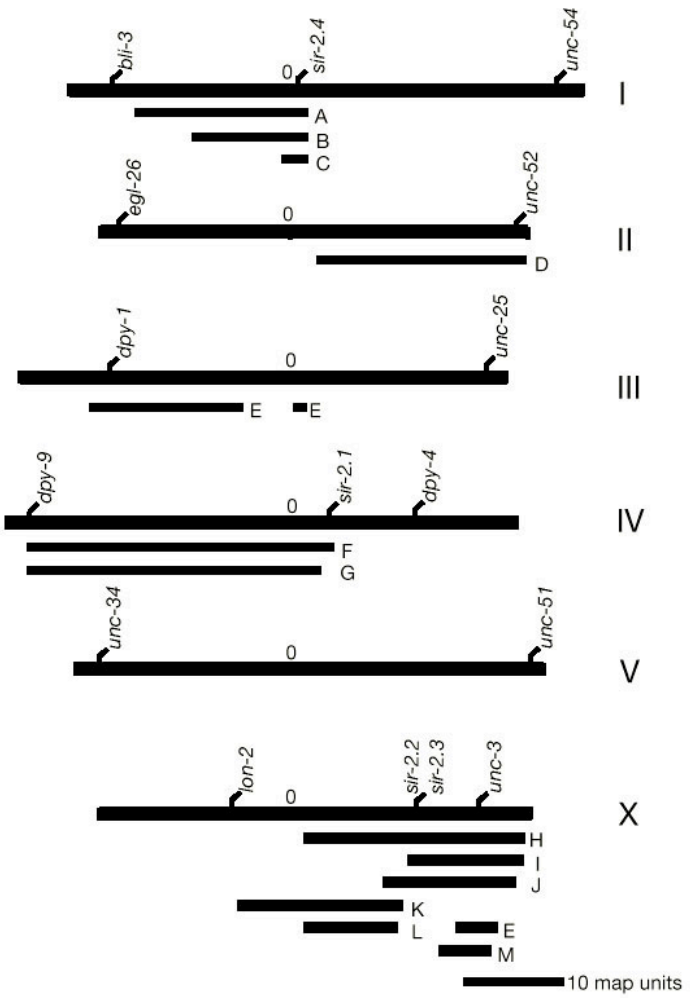


Figure 5.17: Genetic map for some of the duplication strains used in the 2001 study. Figure duplicated from (Tissenbaum and Guarente, 2001).

While the strain DR1786 (F) carrying *mDp4,* which contains the *sir-2.1* locus, was long-lived, a similar chromosome IV duplication, *mDp1*, present in the strains DR907 and MT7070 (G) did not increase life span. *mDp1* contains ~90% of the *mDp4* duplication, but excludes the region containing the *sir-2.1* locus.

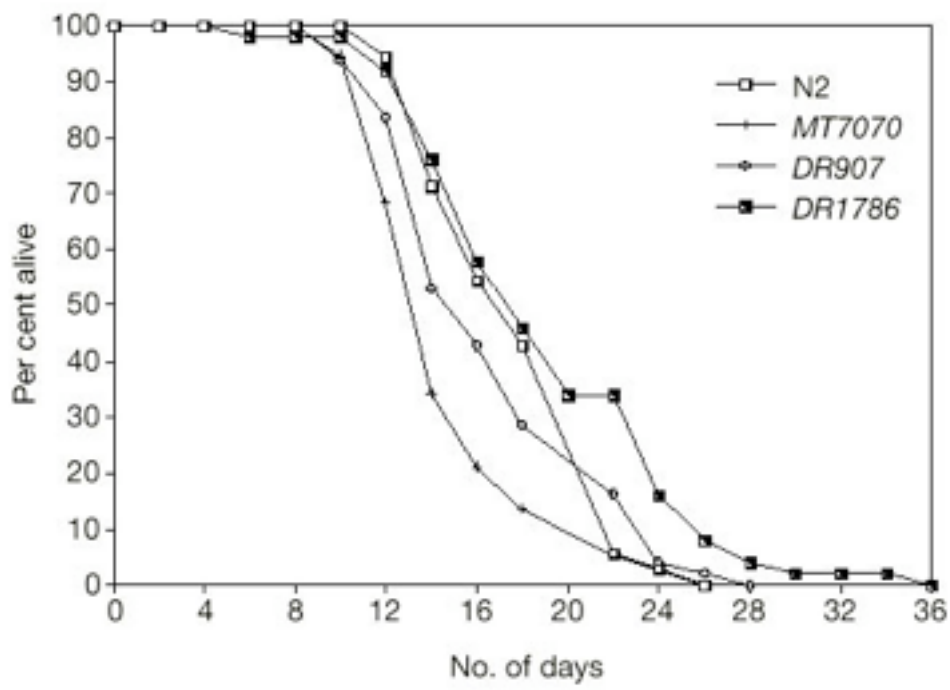


Figure 5.18: Published life span data with the *mDp4* strain DR1786 (Replicated from (Tissenbaum and Guarente, 2001). MT7070, DR907 and N2 wildtype were used as controls.

The published life span trials testing the *mDp4* duplication (DR1786) strain were repeated twice. However, in both cases the wildtype control was somewhat short-lived (15.2±0.5 and 15.1±0.3 days mean life span at 20°C), whereas the duplication strain showed a life span typical of wildtype *C. elegans* (17.3±1.0 and 21.1±1.0 mean life span). The wildtype life span curve presented in the original graph (see Figure 5.18) represents the combined data of 13 single life spans with in total 451 animals. By contrast, the life span curves of the control duplication strains MT7070 and DR907 represent a single trial with 38 and 49 worms, respectively, and the duplication strain DR1786 consists of two single trials with 50 animals in total. The combined data of 13 trials pushes the wildtype mean life span to the more normal 18 days, which obscures the fact that the wildtype control was short-lived in the two trials that included DR1786.

In my trials, N2 animals were used as wildtype controls and life span experiments were performed at 20˚C. One day before the start of the life span experiments FUdR was applied to the plates to an end volume of 10 µM or 40 µM FUdR to prevent progeny production (see Materials & Methods, section 2.2.4.).

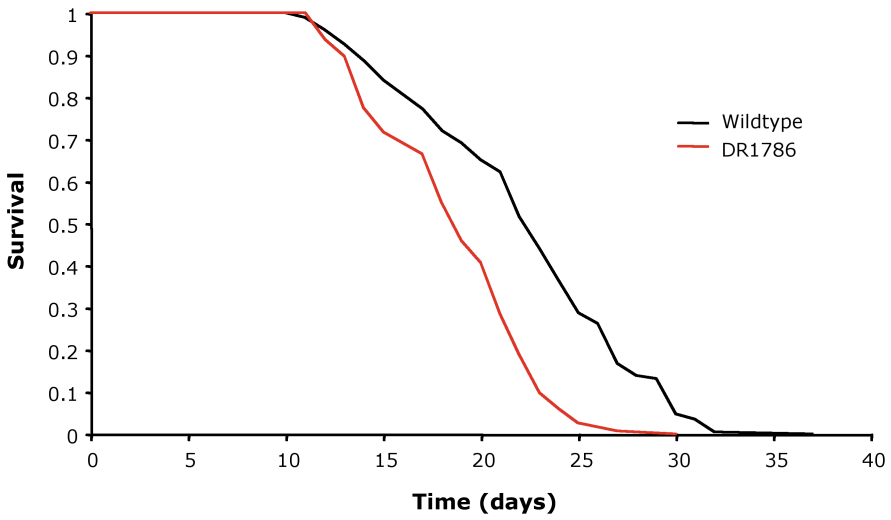


Figure 5.19: Life span of the *mDp4* duplication strain. The trials were performed at 20˚C, with 10 µM FUdR. Genotypes: N2 wildtype, DR1786 *dpy-13(e184) unc-24(e138); mDp4 [unc-17(e245)] (IV;?).* The life span curves represent the combined data of two independent biological replicates. The statistical analysis is presented in Table 5.6.

Table 5.6: Life span of the *mDp4* duplication strain. The trials were performed at 20˚C, with 10 µM FUdR. [C] combined data. Genotypes: N2 wildtype, DR1786 *dpy-13(e184) unc-24(e138); mDp4 [unc-17(e245)] (IV;?). p,* log rank test. [n] Independent biological replicate number.

strain	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* wildtype**	***p vs.* wildtype**
Wildtype	**[C] 318/75**	**22.3**		
	[1] 170/34	22.9		
	[2] 148/41	21.8		
DR1786	**[C] 183/172**	**18.8**	**-15.7**	**<.0001**
	[1] 109/98	18.8	-17.9	<.0001
	[2] 74/74	18.9	-13.3	<.0001

Life span measurements with the duplication strain DR1786 did not show an increase in *C. elegans* life span. Contrary, the strain carrying *mDp4* was twice shorter-lived than wildtype animals (18.8 days *vs.* 22.9 days, respectively, $p<.0001$). However, for reasons unknown in both trials the wildtype control was uncharacteristically long-lived, possibly masking the life span extension of the DR1786 strain. Supporting that view is one biological replicates of my colleague Dr Filipe Cabreiro, showing a slight, but significant increase in life span of the duplication strain (data not shown).

5.2.7.1. Life span of *mDp4* duplication strain is increased upon reduced *sir-2.1* expression

Next, I tested whether any difference in life span between DR1786 and N2 is *sir-2.1* dependent. The original publication attributed the increase in life span of this strain to its extra copy of *sir-2.1*. If correct, then decreased *sir-2.1* expression should decrease the life span of this strain. The animals were maintained again for two generations on the *sir-2.1* RNAi or control RNAi prior to the life span measurements. N2 wildtype worms were used as control. To avoid offspring, FUdR was applied topically one day before in a final concentration of 10 µM.

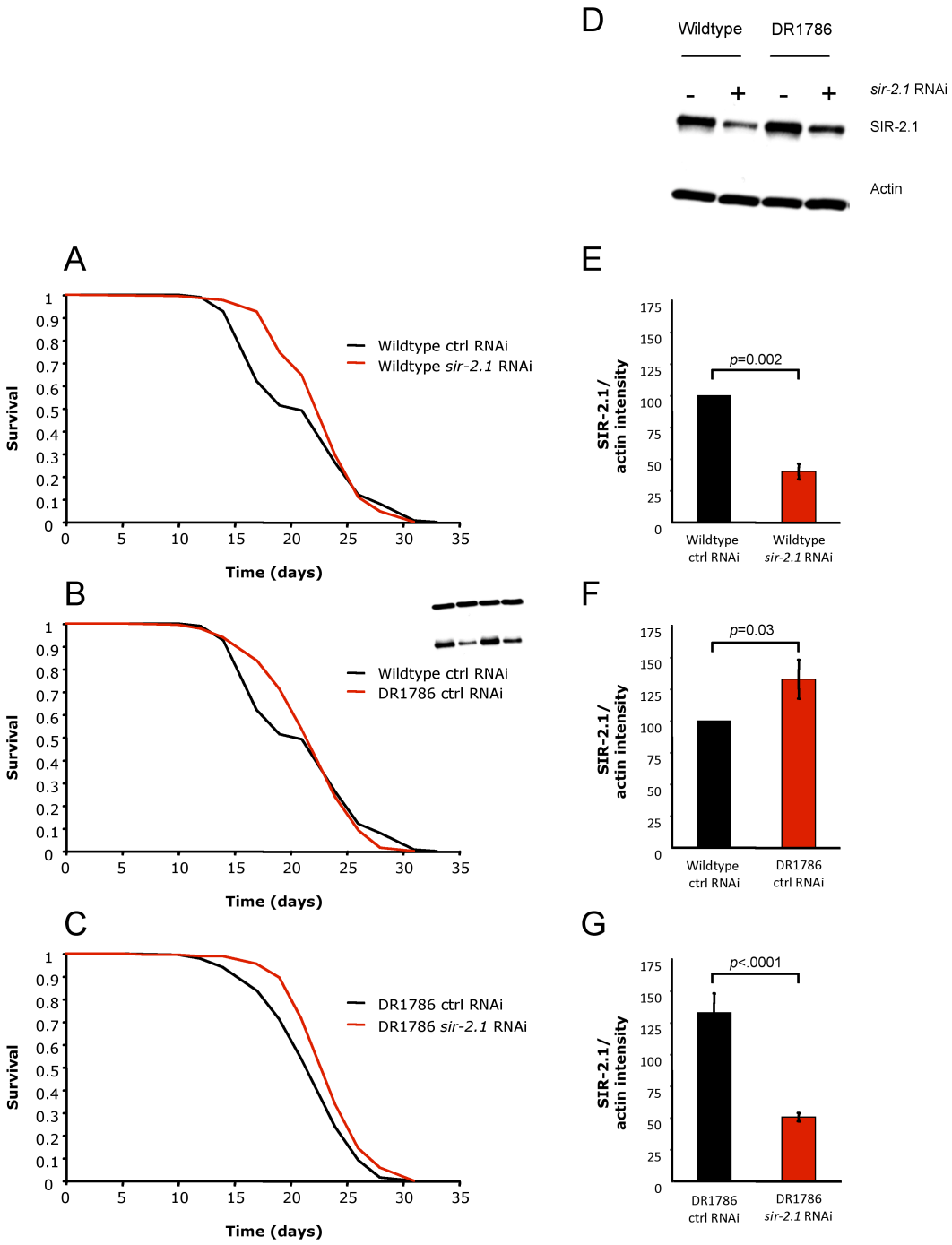


Figure 5.20: Effect of *sir-2.1* RNAi on life span of DR1786. The trials were performed at 20˚C, with 10 µM FUdR. Genotypes: N2 wildtype, DR1786 *dpy-13(e184) unc-24(e138); mDp4 [unc-17(e245)] (IV;?).* **(A-C)** Life span curves of both strains on control and *sir-2.1* RNAi. **(D)** Representative western blot performed by Dr Filipe Cabreiro. **(E-G)** The corresponding western blot quantification. Each column represents four biological replicates and the statistic analysis was performed with the Student's T-test. The error bars show the S.E.M. The life span curves represent the combined data of two independent biological replicates, one performed by Dr Filipe Cabreiro, one by myself. The statistical analysis is presented in Table 5.7.

Table 5.7: Effect of *sir-2.1* RNAi on life span of DR1786. The trials were performed at 20˚C, with 10 µM FUdR. Ctrl RNAi is the L4440 plasmid vector (negative control). Genotypes: N2 wildtype, DR1786 *dpy-13(e184) unc-24(e138); mDp4 [unc-17(e245)] (IV;?). p,* log rank test. [n] Independent biological replicate number. Trial [2] was performed by Dr Filipe Cabreiro.

Strain	**RNAi**	**Deaths/ censored**	**Mean life span (days)**	**% *vs.* wildtype ctrl**	***p vs.* wildtype ctrl**	**% *vs.* DR1786 ctrl**	***p vs.* DR1786 ctrl**
Wildtype	Ctrl	**[C] 295/12**	**20.3**				
		[1] 175/7	21.5				
		[2] 120/5	18.6				
Wildtype	*sir-2.1*	**[C] 262/58**	**21.2**	**+4.4**	**0.16**		
		[1] 117/49	23.1	+7.4	0.02		
		[2] 145/9	19.3	+3.8	0.27		
DR1786	Ctrl	**[C] 330/55**	**22.3**	**+9.9**	**0.0005**		
		[1] 170/41	22.2	+3.3	0.92		
		[2] 160/14	22.5	+21	<.0001		
DR1786	*sir-2.1*	**[C] 288/70**	**23.8**	**+17.2**	**<.0001**	**+6.7**	**0.0001**
		[1] 147/55	23.9	+11.2	0.003	+7.7	0.0005
		[2] 141/15	23.7	+27.4	<.0001	+5.3	0.01

My wildtype control was again uncharacteristically long-lived, and defined a slight, but no significant increase in life span of the duplication strain (21.5 days *vs*. 22.2 days, respectively, p=0.92). However, Filipe Cabreiro's N2 control showed a characteristic life span of 18.6 days, revealing a significant increase in life span of the *mDp4* strain of 21% (p<.0001). Our combined data supports the reported life span extension of this strain (see Table 5.7).

However, the life span of DR1786 is increased by 6.7% on *sir-2.1* RNAi compared to its control (23.8 days *vs.* 22.3 days, respectively, p=0.0001). This implies that the increased life span of this strain is not *sir-2.1* dependent. Furthermore, from this and earlier trials it seems that reduced *sir-2.1* expression is even beneficial, increasing the life span of DR1786 even further.

5.3. Discussion

5.3.1. LG100 longevity is attributable to *dyf-?(wu250) I*

From the results shown in this chapter, it is clear that the reason for the extended life span of LG100 is not the *sir-2.1* over-expression, but rather a second site mutation causing a dye-filling defective phenotype.

LG100 was generated via microinjection. This technique creates transgenic animals with large extrachromosomal DNA arrays that are unstable in cell division and inherited either in a mosaic pattern or not at all (Mello et al., 1991). To get stable lines, the extrachromosomal DNA array needs to be integrated into the chromosome of the animals. In the case of LG100, gamma irradiation was used to cause double-strand breaks in the chromosomes, which allowed ligation of the DNA arrays into the chromosomes during DNA repair.

Gamma irradiation can also cause second site mutations in one or more genes (Riddle, 1997). Therefore it is necessary to standardize the background of the transgenic animal by out-crossing it, ideally at least five times, to a N2 wildtype background. Unusually, LG100 was not out-crossed prior to the experiments published in the cited papers. Work presented here shows that backcrossing abolishes the increase in life span without changing the *sir-2.1* expression at the mRNA or protein levels. This indicates that *sir-2.1* over-expression alone is not responsible for the extended life span in the LG100 strain. A post-doc in the laboratory of M. Kaeberlein's (University of Washington, USA), G. L. Sutphin, repeated the out-crossing of LG100 and life span tests, with the same outcome (Burnett et al., 2011).

Furthermore, the reduction of *sir-2.1* expression levels via RNAi showed no effect on life span in LG100 (Table 5.4), confirming that the increased life span of LG100 is not *sir-2.1*-dependent.

5.3.2. *E. coli* food source influences effect of *dyf-?(wu250)* on life span

The reason that LG100 was not as long-lived on the RNAi bacteria HT115 as on OP50 might be the difference in *E. coli* food source. It is well established that different *E. coli* food sources can influence life span in *C. elegans* (Garsin et al., 2003; Maier et al., 2010). The difference between OP50 and HT115 lies in the *E. coli* strains used to generate both. OP50 originates from an *E. coli* B strain (Brenner, 1974), whereas HT115 comes from an *E. coli* K-12 strain (Dasgupta et al., 1998; Takiff et al., 1989). Several recent studies have shown differences in *C. elegans* life span when grown on OP50 *versus* HT115 (Howard et al., 2000; Maier et al., 2010).

Mutations in genes responsible for cilia formation in sensory neurons, such as *osm-3* and *daf-10,* result in Dyf and Age phenotypes (see Introduction, section 1.4.8.) and show food-dependent effects on life span. Mutations in *daf-10*, for instance, result in increased life span on both, OP50 and HT115, whereas mutations in *osm-3* increase life span on OP50 only, as in the case of LG100. This indicates that sensory neurons mediate the effect of different food sources on *C. elegans* life span, supporting an earlier study showing that gustatory or olfactory neurons can increase or decrease *C. elegans* life span (Alcedo and Kenyon, 2004). The food-dependent decrease or increase in life span is reported to be determined by the type of *E. coli*

lipopolysaccharide structure (Maier et al., 2010). Considering S. S. Lee's and our data that LG100 has a second site mutation resulting in a Dyf phenotype, which affects sensory neurons, it is not surprising to see a food-source dependent effect on life span in this strain.

5.3.3. Variability among *rol-6* control lines in life span

Since this part of my thesis shows the importance of standardizing the genetic background and use of appropriate controls, I wanted to discuss the observation about the *rol-6* control line GA707 and about this phenotypic marker in general. The strain GA707 showed a high variation in life span in the different trials (see Table 5.1 and 5.2). In some trials this *rol-6* line was significantly longer-lived, significantly shorter-lived or showed no difference compared to the N2 wildtype control. Variations within a strain over time are not unheard of. One reason for such changes is spontaneous mutations occurring in a single individual that gets passed on to the progeny (Partridge and Gems, 2007). A further possibility is heritable epigenetic alterations, and repetitive DNA such as in transgene arrays is subject to epigenetic effects. Since the trials are in chronological order, the possibility that a spontaneous mutation occurred in this strain affecting the life span can be excluded. Instead, there may be an issue with the *rol-6* marker. *C. elegans* strains carrying this phenotypic marker sometimes show large differences in life span between lines (personal communication with D. Riddle).

5.3.4. Conclusions

The data presented here shows that the increase in life span in the high-copy number *sir-2.1* over-expressing line LG100 is caused by a second site mutation. It also implies that *sir-2.1* over-expression does not increase life span. However, the prior study also reports increases in life span in the extrachromosomal predecessor lines, bearing *geEx1*, *geEx2* and *geEx3* (Tissenbaum and Guarente, 2001). Clearly, this is not attributable to mutagenic effects gamma irradiation. The findings presented here suggest that the increase in life span of the extrachromosomal predecessors is unlikely to be caused by *sir-2.1* over-expression. However, none of these lines were used in follow up studies reporting the *sir-2.1*-dependent increase in *C. elegans* life span, and they are not available via the CGC. Rather than investigate these lines I instead focused on the other widely used *sir-2.1* over-expression line, NL3909 (Viswanathan et al., 2005). Analysis of that strain is subject of the next chapter.

Chapter 6: The effect of *sir-2.1* over-expression on *C. elegans* life span in the transgenic line NL3909

6.1. Introduction

Four years after the 2001 study stating that over-expression of *sir-2.1* extends *C. elegans* life span, there appeared a new study reporting a role for *sir-2.1* in the regulation of stress response genes (Viswanathan et al., 2005). For this study a new *sir-2.1* over-expressing strain was generated, and this was reported to be long-lived.

6.1.1. Generation of a new *sir-2.1* over-expressing line

sir-2.1 is predicted to be the downstream gene of a two-gene operon (CEOP4372). During the generation of the high-copy *sir-2.1* over-expresser line LG100 (see Chapter 5) the regulatory region for CEOP4372 was not included in the transgene used for the microinjection (Tissenbaum and Guarente, 2001). A new *sir-2.1* over-expressing line was created by M. Viswanathan (Guarente lab), which included the entire CEOP4372 operon. This strain, NL3909, is a low copy *sir-2.1* over-expressing strain generated by microbombardment. CEOP4373, with the complete *sir-2.1* sequence plus 2.5kb of upstream sequence including the gene R11A8.5, encoding a glutathione S-transferase-related protein, was subcloned into the vector pRP2510 (Berdichevsky et al., 2006). This construct was introduced into *unc-119(ed3)* worms. *unc-119* is necessary for development of the nervous system and normal movement and feeding behaviour. Worms with mutations

in this gene are uncoordinated (Unc). *unc-119(ed3)* contains a point mutation, which is a c to t substitution.

The transgene array in NL3909 line carries, besides the extra copies of *sir-2.1*, wildtype copies of the plasmid pRP2519 *(unc-119[+])*, rescuing the *unc-119* mutant phenotype. The presence of the transgene array was followed by selecting wildtype animals (i.e. rescue of *unc-119(ed3)*) and verified via PCR. Simultaneously a control strain, NL3908, was generated carrying the same co-transformation rescue marker, but without *sir-2.1* transgenes. The life span of both strains were measured in a single trial, on NGM/DMSO plates at 20°C with FUdR (DMSO concentration is not stated but it was found to have no effect on life span), showing a mean life span of 27.0 days for NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+) sir-2.1(+)]*, and 21.4 days for its control strain NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)]*. The extension of this *sir-2.1* over-expression line was, as in LG100, *daf-16* dependent. This was again shown by crossing the *pkIs1642* transgene array into a *daf-16* background (Viswanathan et al., 2005).

6.1.2. Effect of *sir-2.1* over-expression in *C. elegans*

The 2005 study reported that *sir-2.1* extends life span by repressing expression of ER stress genes. Here the supposed sirtuin activator resveratrol was used. The report acknowledged but afterwards dismissed the then recent findings questioning that resveratrol is a sirtuin activator (Borra et al., 2005; Kaeberlein et al., 2005b), giving more credence to earlier findings supporting the sirtuin-activating role of resveratrol (Howitz et al., 2003; Wood et al., 2004) (see Introduction, section 1.6.4.).

Wildtype and *daf-16(mgDf50)* mutant animals showed a dose-dependent increase in life span upon resveratrol treatment, with 1 mM producing the largest effect. On the other hand, LG231 *sir-2.1(ok434)*, NL4251 *sir-2.1(pk1640::Tc1)* and LG267 *daf-16(mgDf50)*; *sir-2.1(pk1640::Tc1)* mutants showed no increase in life span upon resveratrol treatment. These results further supported the view that effect of resveratrol on life span involves SIR-2.1 activation. Here it is puzzling that *daf-16* is necessary for life span increase by *sir-2.1* over-expression (Tissenbaum and Guarente, 2001), but not by resveratrol. To explain this the authors proposed an involvement in a different longevity pathway like the IIS one (see Introduction, section 1.6.2.4.1.). Both NL3909 and its control strain NL3908 showed extended life span upon resveratrol treatment. The additive effect of resveratrol and *sir-2.1* over-expression supported the idea that resveratrol and *sir-2.1* over-expression affect *C. elegans* life span via different mechanisms. This study also presented evidence that *sir-2.1* is a negative regulator of expression of ER stress genes, whereas resveratrol activated these genes, resulting in an extended life span. Surprisingly, this result implies an inhibiting effect of resveratrol on sirtuin activity instead of the previously assumed activating effect (Viswanathan et al., 2005).

In 2006, the same group published a new study offering another possible mechanism for the effect of *sir-2.1* on life span. Here, *sir-2.1* is reported to interact with 14-3-3 proteins, resulting in *daf-16* activation and extended life span (Berdichevsky et al., 2006). In this study, *sir-2.1* RNAi was reported to suppress the extended life span of the NL3909 and also its resistance to heat stress. The intrinsic thermotolerance of the NL3909 strain

was recently reconfirmed (Burnett et al., 2011) (see Discussion). Notably, the study in 2006 only shows *sir-2.1* over-expression data for LG100; This far *sir-2.1* over-expression in NL3909 has not been demonstrated (Berdichevsky et al., 2006).

In the previous chapter, I showed that *geIn3* does not increase *C. elegans* life span. This is not consistent with the observation that *pkIs1642* increases life span. One possibility raised by the previous chapter is that the longevity of NL3909 is not, in fact attributable to *pkIs1642*, but rather to background effects. I therefore set out to test this possibility.

6.2. Results

6.2.1. The *sir-2.1* over-expressing line NL3909 is long-lived

Given the previous controversies about sirtuins and ageing, and our findings with the older *sir-2.1* over-expressing strain LG100 (see Chapter 5), we decided to verify that *sir-2.1* over-expression increases life span in NL3909. The first step was to verify the longevity of NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+) sir-2.1(+)]*. As already mentioned, there are two published life span trials showing that NL3909 is long-lived, one on *E. coli* OP50 (on NGM plates with DMSO, as a control for treatment with resveratrol in DMSO (Viswanathan et al., 2005), and one on RNAi control plates with *E. coli* HT115 (Berdichevsky et al., 2006). I therefore performed several repeat trials of this experiment, using both N2 and the co-transformation marker strain NL3908 as negative controls. The life span experiments were performed at 20°C on *E. coli* OP50. One day before the start of the life span trial, I applied FUdR topically to the plates to a final concentration of either 10 µM or 40 µM (see Materials & Methods, section 2.2.4.).

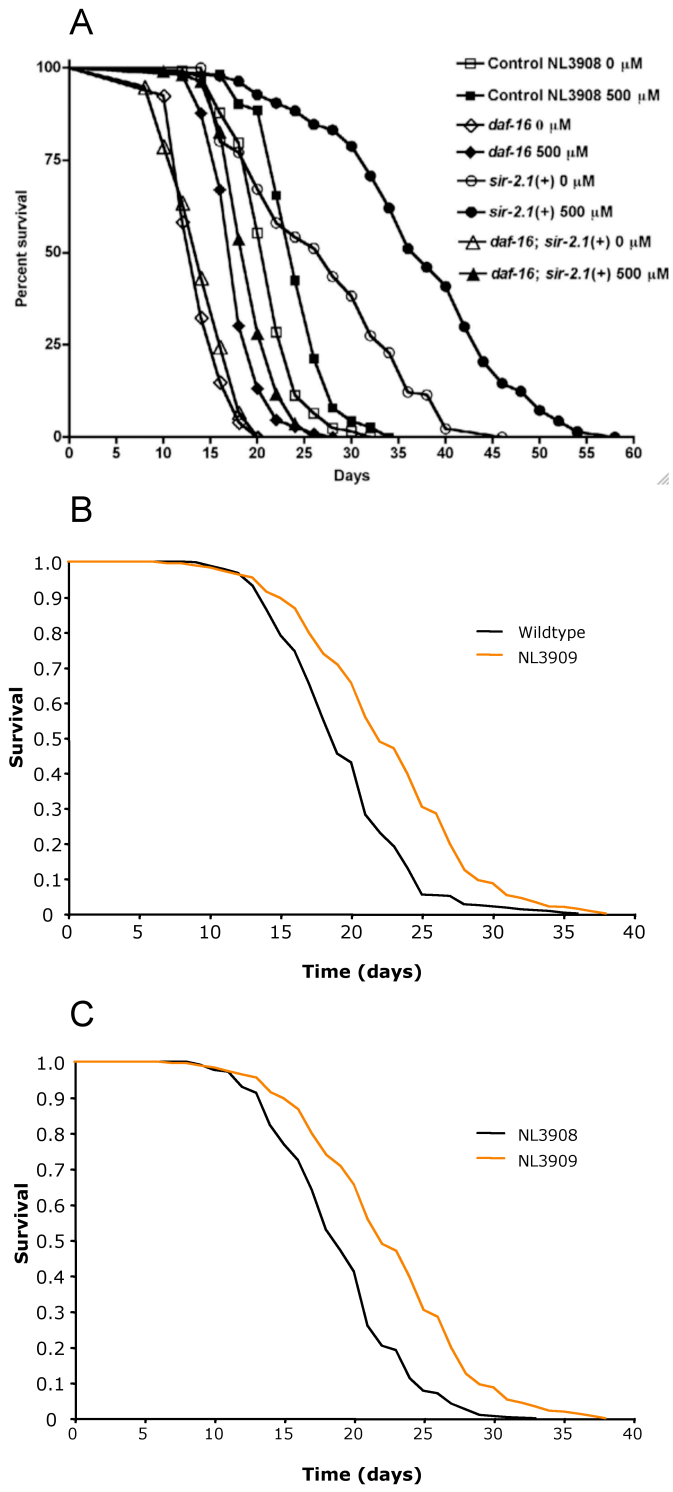


Figure 6.1: Life span effect of *sir-2.1* over-expression in NL3909. (A) Original life span trial with NL3909 (reproduced from (Viswanathan et al., 2005). **(B,C)** Genotypes: N2 wildtype, NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)]),* NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)]).* Life span curves of NL3909 compared to wildtype and its genetic control. The life span curves represent the combined data of six independent biological replicates. The statistical analysis is presented in Table 6.1.

Table 6.1: Life span effect of *sir-2.1* over-expression in NL3909. Trials were performed at 20°C with 10 µM ([1]-[3]) or 40 µM ([4]-[6]) FUdR. Genotypes: N2 wildtype, NL3908 *unc-119(ed3) III (pkIs1641[unc-119(+)]),* NL3909 *unc-119(ed3) III (pkIs1642[unc-119(+); sir-2.1(+)]).* [C] combined data, , *p*, probability of being the same as specified control (log rank). [n] biological replicate number

Strain	Deaths/ censored	Mean life span (days)	% *vs.* wildtype	*p vs.* wildtype	% *vs.* genetic ctrl	*p vs.* genetic ctrl
Wildtype	**[C] 507/368**	**19.5**				
	[1] 58/68	17.1				
	[2] 90/28	17.4				
	[3] 106/19	19.4				
	[4] 84/41	18.8				
	[5] 79/48	19.9				
	[6] 90/162	22.6				
NL3908	**[C] 530/107**	**19.2**	**-1.5**	**0.30**		
	[1] 113/19	19.9	+16.4	<.0001		
	[2] 118/10	17.3	-0.6	0.89		
	[3] 99/19	17.1	-11.9	0.003		
	[4] 104/21	20.1	+6.9	0.09		
	[5] 96/38	21.4	+7.5	0.0007		
	[6] 28/99	22.4	-0.9	0.31		
NL3909	**[C] 619/130**	**22.6**	**+15.9**	**<.0001**	+17.7	**<.0001**
	[1] 114/13	21.9	+28.1	<.0001	+10.1	0.0004
	[2] 117/11	21.5	+25.3	<.0001	+24.3	<.0001
	[3] 110/9	21.8	+ 12.4	0.0006	+27.5	<.0001
	[4] 119/5	23.3	+23.9	<.0001	+15.9	<.0001
	[5] 114/12	24	+20.6	<.0001	+12.1	<.0001
	[6] 45/80	24.2	+7.1	0.09	+8	0.02

My findings robustly confirm the reported longevity of the low copy number *sir-2.1* over-expressing strain NL3909 compared to control animals. The NL3909 strain was not quite as long-lived as previously reported (published increase of 26.2% *vs.* 17.1% here). This could reflect minor inter-lab differences in culture conditions. The NL3908 control strain was longer-lived than N2 in two of six trials ([1] and [5], $p<.0001/0.0007$), but shorter-lived in a third ([3], $p=0.003$). However, summed data suggests no real difference between the two negative controls.

6.2.2. Effect of backcrossing on the *sir-2.1* over-expresser strain NL3909

After finding that backcrossing in the first *sir-2.1* over-expressing line LG100 abolished the increase in life span, I wanted to check that the same was not true for the NL3909 strain. NL3909 and NL3908 were out-crossed six times using the *unc-119(ed3)* strain HT1593. The rescue of the Unc mutant phenotype by the wildtype *unc-119* allele was used to follow the transgene arrays (see Figure 6.2).

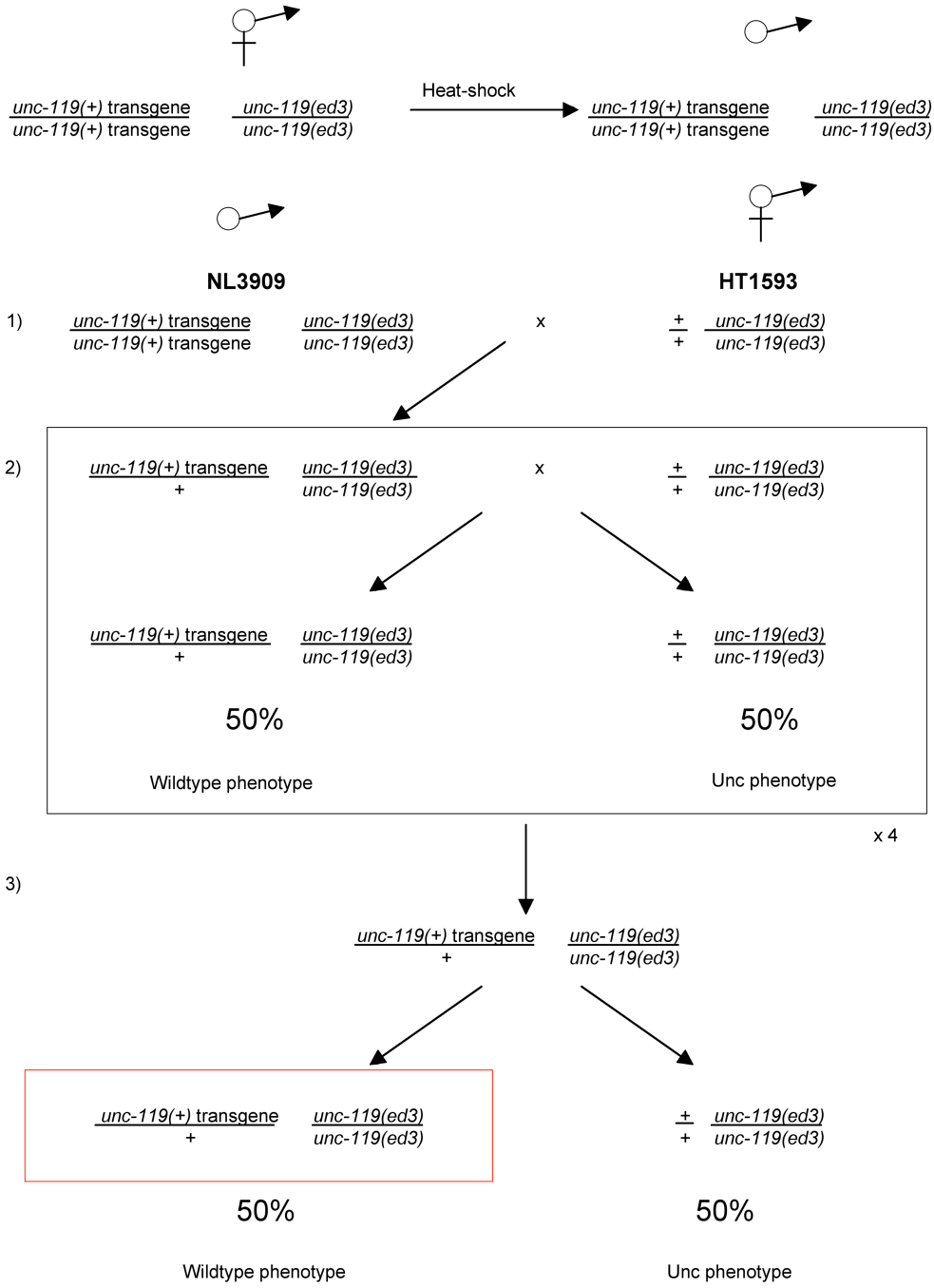


Figure 6.2: The out-crossing scheme of NL3909. First NL3909 was heat-shocked to get males. **1)** The NL3909 males were crossed with HT1593 hermaphrodites, the F_1 generation selfed and **2)** transgenic males, identified by their N2 wildtype phenotype, were again crossed with HT1593 hermaphrodites. The F_2 generation should segregate 50% wildtype animals and 50% phenotypical Unc animals. The step **2)** was repeated four more times. **3)** Phenotypical wildtype animals were picked after the last out-crossing step from the F_2 generation and selfed. Phenotypical N2 wildtype worms were considered as stable line and named GA905. The same out-crossing scheme was used for NL3908. The out-crossed derivative of this strain was called GA906.

The life spans of the out-crossed derivatives of NL3909 and NL3908, GA905 and GA906, respectively, were measured with both parental strains and N2 as controls. The life span measurements were performed at 20˚C, using either 10 μM or 40 μM FUdR to prevent progeny production (see Materials & Methods, section 2.2.4.).

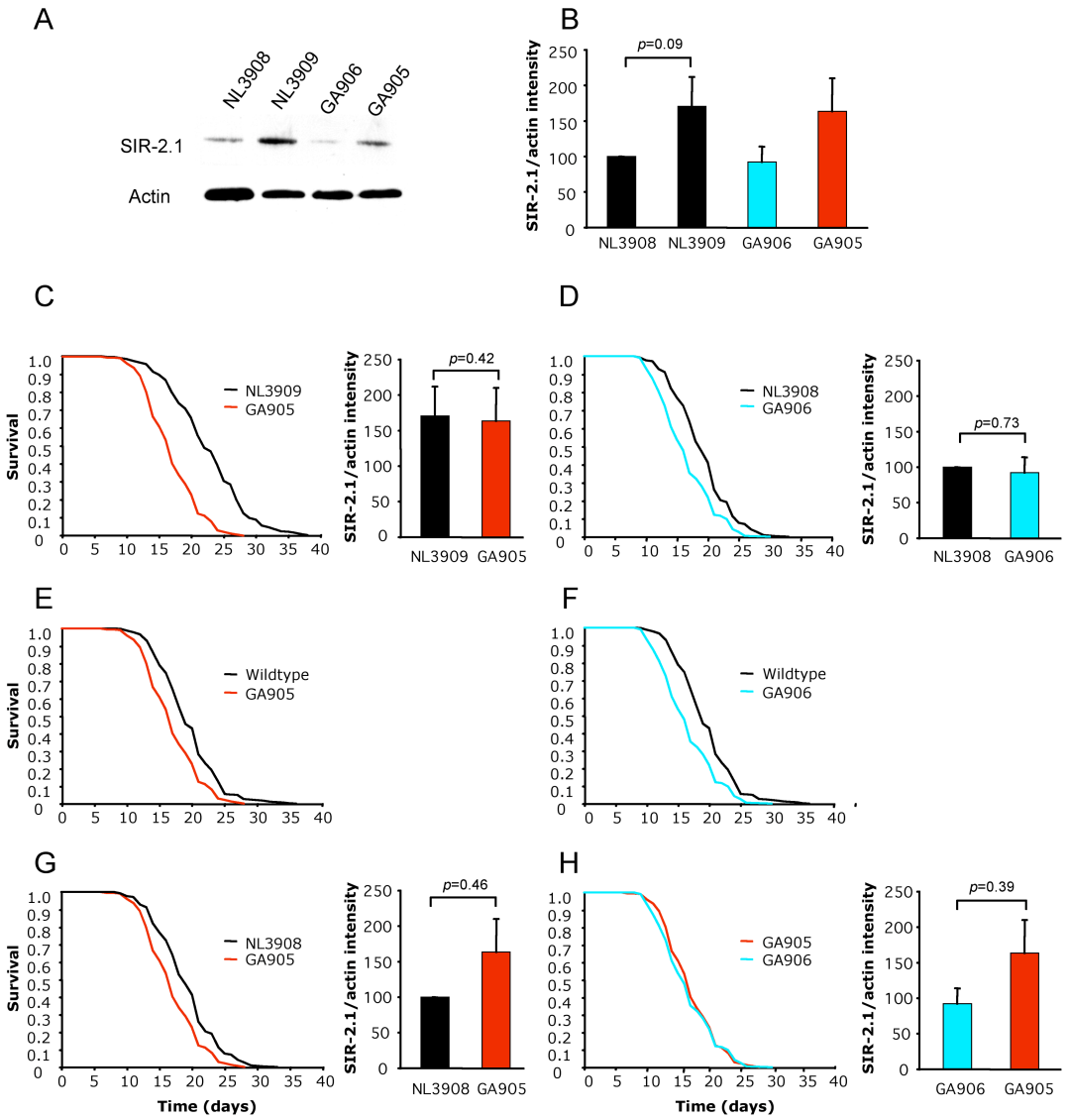


Figure 6.3: Effect of out-crossing on life span in NL3909. Genotypes: N2 wildtype, NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)],*out-crossed derivative GA906 *unc-119(ed3) III (pkIs1641 [unc-119(+)],* NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)],* out-crossed derivative GA905 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)].* **(A, B)** Shows a representative Western blot done by Dr Filipe Cabreiro and the corresponding quantification of the SIR-2.1 levels of four biological replicates. The error bars show the S.E.M. (Burnett et al., 2011). **(C-H)** Life span curves represent the combined data of at least three independent biological replicates. The statistical analysis is presented in Table 6.2.

Table 6.2: Effect of out-crossing on life span in NL3909. The Trials were performed at 20˚C with 10 µM ([1]-[3]) or 40 µM ([4]-[6]) FUdR. Trial [wF] was without FUdR. [C] combined data, *p*, log rank test. Genotypes: N2 wildtype, NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)])*, GA906 *unc-119(ed3) III (pkIs1641 [unc-119(+)])*, NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)]*, GA905 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)].*

Strain	Deaths/ censored	Mean life span (days)	% *vs.* wildtype	*p vs.* wildtype	% *vs.* NL3909	*p vs.* NL3909	% *vs.* NL3908	*p vs.* NL3908	% *vs.* GA905	*p vs.* GA905
Wildtype	**[C] 507/368**	**19.5**								
	[1] 58/68	17.1								
	[2] 90/28	17.4								
	[3] 106/19	19.4								
	[4] 84/43	18.8								
	[5] 79/48	19.9								
	[6] 90/162	22.6								
	[wF] 104/82	20.3								
NL3909	**[C] 619/130**	**22.6**	**+15.9**	**<.0001**						
	[1] 114/13	21.9	+28.1	<.0001						
	[2] 117/11	21.5	+23.6	<.0001						
	[3] 110/9	21.8	+12.4	0.0006						
	[4] 119/5	23.3	+23.9	<.0001						
	[5] 114/12	24	+20.6	<.0001						
	[6] 45/80	24.2	+7.1	0.09						
NL3908	**[C] 530/107**	**19.2**	**-1.5**	**0.30**	**-15**	**<.0001**				
	[1] 113/19	19.9	+16.4	<.0001	-9.1	0.0004				
	[2] 118/10	17.3	-0.6	0.89	-19.5	<.0001				
	[3] 99/19	17.1	-11.9	0.003	-21.6	<.0001				
	[4] 104/21	20.1	+6.9	0.09	-13.7	<.0001				
	[5] 96/38	21.4	+7.5	0.0007	-10.8	<.0001				
GA905	**[C] 291/88**	**17**	**-12.8**	**<.0001**	**-24.8**	**<.0001**	**-11.5**	**<.0001**		
	[1] 91/39	16.1	-5.8	0.01	-26.5	<.0001	-19.1	<.0001		
	[2] 97/23	17.6	+1.1	0.53	-18.1	<.0001	+1.7	0.53		
	[3] 103/26	17.3	-10.8	0.003	-20.6	<.0001	+1.2	0.80		
	[wF] 142/24	19.1	-5.9	0.08						
GA906	**[C] 275/105**	**16.6**	**-14.9**	**<.0001**	**-26.5**	**<.0001**	**-13.5**	**<.0001**	**-2.4**	**0.40**
	[1] 77/47	15.9	-7	0.004	-27.4	<.0001	-20.1	<.0001	-1.2	0.58
	[2] 86/45	16.8	-3.4	0.53	-21.9	<.0001	-2.9	0.63	-4.5	0.32
	[3] 112/13	16.9	-12.9	0.001	-22.5	<.0001	-1.2	0.76	-2.3	0.60
	[wF] 131/31	19	-6.4	0.14					-0.5	0.62

Surprisingly, out-crossing of the *sir-2.1* over-expressing strain NL3909 abolished the increase in life span (Figure 6.3 and Table 6.2). The out-crossed derivatives of NL3909 and its control NL3908 were both significantly shorter-lived than wildtype animals ($p<.0001$) and did not show any difference in life span compared to each other ($p=0.40$). This result was unexpected, since microbombardment should integrate the transgene into the genome without mutagenic effects. At our request, M. Somogyvári, a student in C. Soti's lab at Semmelweis University in Budapest, repeated the out-crossing of these transgenic animals with the same result, i.e. out-crossing of NL3909 abrogated longevity. This suggests that in NL3909, as in LG100, the extended life span is not a function of *sir-2.1* over-expression.

There was no significant difference between the SIR-2.1 protein levels (Western blots by Dr Filipe Cabreiro) in any of the samples (Figure 6.3, A, B). However, the Western blots show a clear trend towards an increase, and an apparent ~60% increase in protein level. Moreover, the out-crossed derivative show a similar apparent increase in SIR-2.1 levels as in the parental strain. This is consistent with the view that this strain is a low-copy over-expresser, as expected from the use of microbombardment to prepare the transgenic lines.

6.2.3. GA905 is competent for life extension

That out-crossing of NL3909 removes its longevity suggests that the longevity of this strain is caused by a genetic factor or factors other than the *pkIs1642* transgene array. However, there are alternative possible explanations. One is that out-crossing has introduced a suppressor mutation that masks the effect of *pkIs1642* on life span. Another is that during subculture, NL3909 has acquired a suppressor mutation closely linked to *pkIs1642*. To probe this possibility, we verified that GA905, the out-crossed derivative of NL3909 is still competent for life extension by reduced IIS. As before, GA905 was maintained for two generations on control and *daf-2* RNAi cultures prior the start of life span measurements to ensure efficient reduction of *daf-2* expression levels. One day prior the start of life span measurements, FUdR was applied topically to the life span plates to a final concentration of 10 µM to prevent development of offspring (see Materials & Methods 2.2.4.).

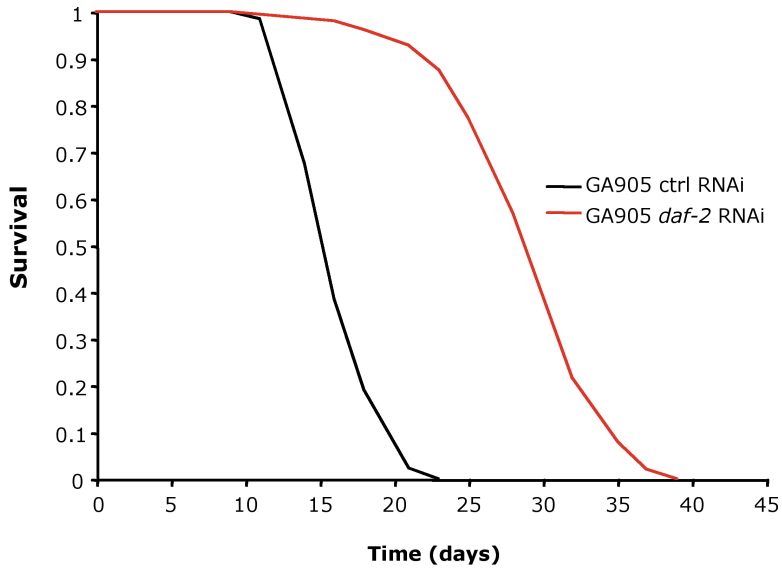


Figure 6.4: Effect of reduced *daf-2* expression on life span in GA905. Genotype: GA905 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)]*

Table 6.3: Effect of reduced *daf-2* expression on life span in GA905. The trial was performed at 25˚C with 10 µM FUdR. Genotype: GA905 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)].* Ctrl RNAi is the L4440 plasmid vector. *p*, log rank test.

Strain	RNAi	Deaths/ censored	Mean life span (days)	% *vs.* ctrl	*p vs.* ctrl
GA905	Ctrl	130/17	16.7		
GA905	*daf-2*	152/6	29.5	+76.6	<.0001

The *sir-2.1* over-expresser GA905 proved to be robustly long-lived on *daf-2* RNAi. Dr Filipe Cabreiro repeated this trial simultaneously with the same result (data not shown). This provides some evidence against the presence of a suppressor mutation in GA905 that masks effects of *pkIs1642* on longevity. However, it is difficult to entirely rule out the possibility of the presence of a suppressor with selective effects on *sir-2.1* induced longevity. Yet replication of the effects of out-crossing by the Budapest group, and the results with LG100 argue against this interpretation.

6.2.4. Effect of reduced *sir-2.1* expression levels in NL3909

Our backcrossing results imply that the extended life span of NL3909 is not caused by *sir-2.1* over-expression. We attempted therefore to verify this by testing whether reducing *sir-2.1* levels using RNAi suppressed NL3909 longevity. Although it was previously reported *sir-2.1* RNAi abolishes NL3909 longevity (Berdichevsky et al., 2006) (see Figure 6.5) our expectation now was that it would not.

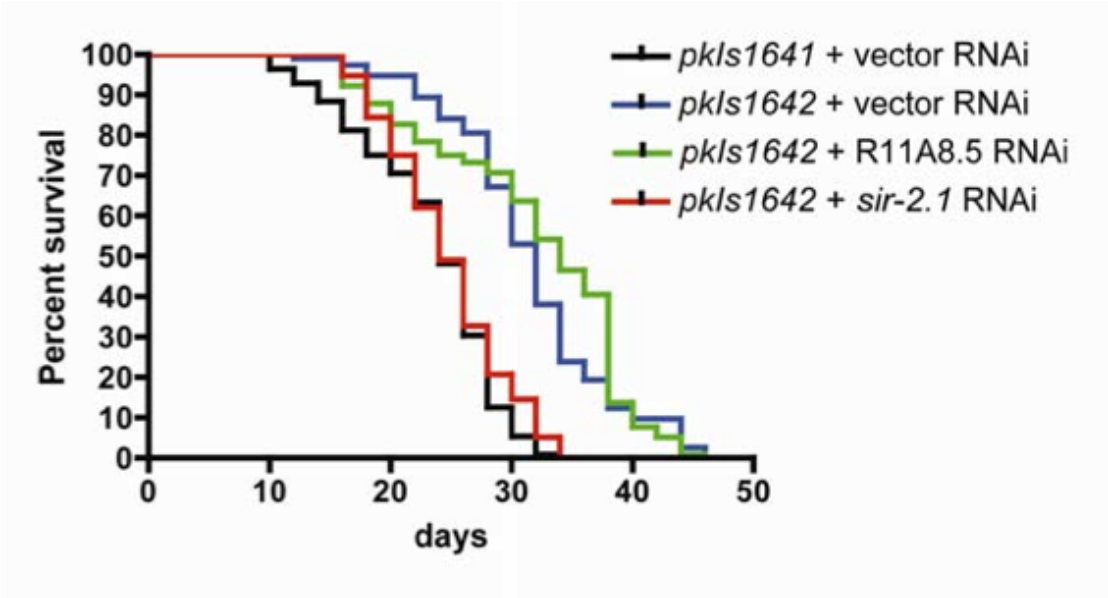


Figure 6.5: Previously reported *sir-2.1* RNAi with NL3909 (reproduced from Berdichevsky et al., 2006, Figure S2 A)**.** The reported median life spans are: NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)])* on vector RNAi 24 days (n=112)*,* NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)]* on vector RNAi 32 days (n=113), on R11A8.5 RNAi 34 days (n=116) and on *sir-2.1* RNAi 24 days (n=115). This trial also shows that the increase in life span is not caused by the R11A8.5 gene included into the transgene. The number of trials and sample sizes are not recorded.

To ensure a reduction in *sir-2.1* expression levels, worms were maintained for two generations on the RNAi cultures prior the start of the life span measurements. The results are presented in Figure 6.6 and Table 6.4.

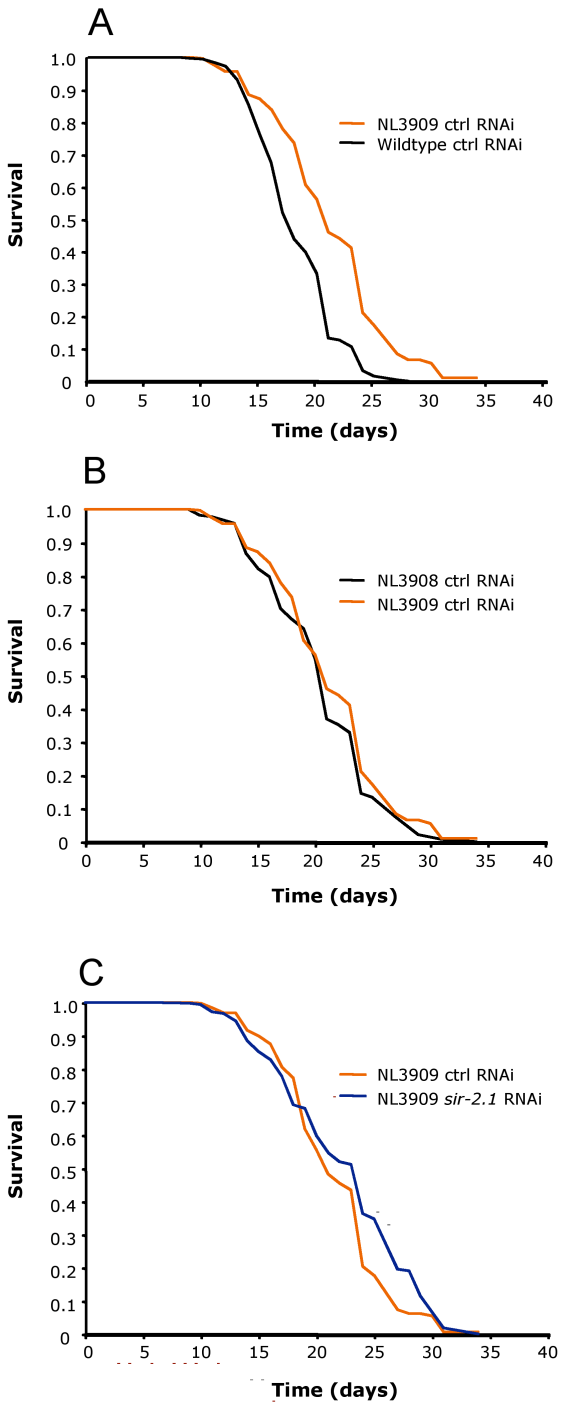


Figure 6.6: Effect of *sir-2.1* RNAi on life span in NL3909. Genotypes: N2 wildtype, NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)],* NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)],*. **(A, B)** Life span curves of NL3909 compared to its control strain NL3908 and N2 wildtype animals. **(C)** Live span of NL3909 on control RNAi L4440 and *sir-2.1* RNAi feeding clones. The life span curves represent the combined data of at least three independent biological replicates. The statistical analysis is presented in Table 6.4.

Table 6.4: Effect of *sir-2.1* RNAi on life span in NL3909. The trials are performed at 20˚C, without FUdR, except for trial [3] where 10 µM FUdR was used. Ctrl RNAi is the L4440 plasmid vector. [C] combined data. L4440 is RNAi plasmid vector (negative control). Genotypes: N2 wildtype, NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)],* NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)]. p*, log rank test. [n] biological replicate number.

Strain	RNAi	Deaths/ censored	Mean life span (days)	% *vs.* wildtype on ctrl	*p vs.* wildtype on ctrl	% *vs.* NL3908 on ctrl	*p vs.* NL3908 on ctrl	% *vs.* NL3909	*p vs.* NL3909 on ctrl
Wildtype	Ctrl	**[C] 319/35**	**18.3**						
		[1] 118/9	17.9						
		[2] 92/2	19.7						
		[3] 109/24	17.7						
Wildtype	*sir-2.1*	**[C] 252/121**	**19**	**+3.8**	**0.05**	**-9.1**	**0.52**		
		[1] 117/8	18.2	+1.7	0.45	-4.1	0.22		
		[2] 49/66	19.1	–3	0.36	+4	0.33		
		[3] 86/47	20.1	+13.6	<.0001	-6.9	0.001		
NL3908	Ctrl	**[C] 370/173**	**20.9**	**+14.2**	**<.0001**				
		[1] 63/60	19.1	+6.7	0.008				
		[2] 78/43	19.9	+1	0.07				
		[3] 115/35	21.6	+22	<.0001				
		[4] 114/35	21.5						
NL3908	*sir-2.1*	**[C] 365/182**	**22**	**+20.2**	**<.0001**	**+5.3**	**0.001**		
		[1] 54/77	17.1	-4.5	0.32	-10.5	0.001		
		[2] 69/54	21.6	+9.6	<.0001	+8.5	0.03		
		[3] 119/25	22.8	+28.8	<.0001	+5.6	0.13		
		[4] 123/26	23.4			+8.8	0.13		
NL3909	Ctrl	**[C] 427/116**	**21.5**	**+17.5**	**<.0001**	**+2.9**	**0.07**		
		[1] 93/33	18.9	+5.6	0.009	-1	0.85		
		[2] 84/44	22.5	+14.2	<.0001	+13.1	0.0009		
		[3] 125/20	22.2	+25.4	<.0001	+2.8	0.88		
		[4] 125/19	22.2			+3.3	0.66		
NL3909	*sir-2.1*	**[C] 261/276**	**22.5**	**+23**	**<.0001**	**+7.7**	**<.0001**	**+4.7**	**0.001**
		[1] 78/46	16.6	-7.3	0.05	-13.1	0.0001	-12.2	0.0002
		[2] 18/100	22.4	+13.7	<.0001	+12.6	0.0005	-0.4	0.42
		[3] 84/65	24.6	+39	<.0001	+13.8	0.0002	+10.8	<.0001
		[4] 81/65	24.2			+12.6	<.0001	+9	0.001

Four life span trials were conducted, with differing results. In one trial [1] *sir-2.1* RNAi reduced NL3909 longevity (p=0.05) as previously reported (Berdichevsky et al., 2006). However, this was not seen in three subsequent trials, where NL3909 lived significantly longer on *sir-2.1* RNAi than on the control RNAi vector (p<0.001) in each case. A similar effect was seen in NL3908. This could imply a negative effect of *sir-2.1* on life span in these strains.

A problem with these trials was that NL3909 was not significantly longer-lived on the control vector RNAi L4440 relative to NL3908 animals on the same bacteria (though it was longer-lived than N2). Thus, it appears that in this lab at least the cause of longevity in NL3909 is sensitive to the *E. coli* strain, since that longevity is not expressed on *E. coli* HT115 RNAi strain, somewhat as already observed for the LG100 strain (see Chapter 5). However, in the case of NL3909 the life span increase is not merely reduced compared to that on *E. coli* OP50 (mean of 22.6 days on OP50 *vs.* 21.5 days on HT115), but it is no longer significant compared to NL3908 (mean of 20.9 days on HT115, p=0.07). This suggests that we cannot draw robust conclusions from the fact that *sir-2.1* RNAi does not decrease life span of NL3909 since it is not longer long-lived on HT115 to begin with. However, on HT115 NL3909 is longer-lived than N2, and NL3909 longevity is unaffected by *sir-2.1* RNAi. One possibility is that HT115 increases life span of NL3908 relative to maintenance on OP50.

6.2.5. Testing NL3909 for a second site *daf-c* mutation

The effects of out-crossing in two independent labs indicate that *sir-2.1* over-expression in the NL3909 is not responsible for its extended life span. One possibility is that NL3909, like LG100, contains a single second site mutation that increases its life span. I tested NL3909 for a Dyf phenotype, but found it to be wildtype for dye filling (data not shown).

A second possibility is that NL3909 and NL3908 contain a second site Daf-c mutation. The strain often used for microbombardment with transformation rescue of *unc-119* is DP38. The genotype of DP38, as described in WormBase is "*unc-119(ed3) III; daf-?*". The "*daf-?*" refers to an unlinked dauer constitutive mutation.

One possibility is that NL3909 and NL908 were created using DP38, and that *daf-?* accounts for NL3909 longevity. Possibly *daf-?* synergizes with *sir-2.1* to increase life span, or background factors suppress the effect of *daf-?* on life span in NL3908. Note that NL3908 is as long-lived as NL3909 when cultured on HT115, and is also long-lived on OP50 in some trials. To test for the presence of *daf-?* in NL3909 and NL3908, I performed high temperature induction of constitutive dauer formation (Hid) assays. Hid animals have increased dauer formation at temperatures above 25˚C, usually 27˚C (Malone et al., 1996). One cause of Hid is reduced IIS and increased DAF-16 activity. In particular, I wanted to know: is NL3909 Hid and is the Hid trait removed by backcrossing?

The Hid phenotype was assayed by performing a brief egg lay for 2-3h followed by incubation of the progeny at 20˚C and 27˚C. 44-55h later the frequency of dauer formation was counted in the progeny on the plates kept at

27˚C. The progeny at 20˚C served as a negative control. The IIS mutant strain TJ1052 *age-1(hx546)* cultured at 27˚C was used as a positive control, since this strain is Hid. GR1307 *daf-16(mgDf50)* was used as a negative control at 27˚C, since this strain is dauer-defective (see Materials & Methods, section 2.2.6.3.). The Hid assay is notorious for high variation between trials. Only a difference of 0.5˚C in temperature can have a large effect on the frequency of dauer formation in each strain at temperatures near 27˚C. For example, temperature differences from 26.5˚C to 27.1˚C can cause a shift from 60% to 100% dauer formation (Ailion and Thomas, 2000).

Table 6.5: Hid assay of NL3909 and NL3908 and their out-crossed derivatives. Genotypes: N2 wildtype, TJ1052 *age-1(hx546) II*, GR1307 *daf-16(mgDf50) I*, NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)])*, GA905 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)])*, NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)])*, GA906 *unc-119(ed3) III (pkIs1641 [unc-119(+)]),* DP38 *unc-119(ed3) III; daf-?.* df= dauer formation, pdf= partial dauer formation. (n) number of animals. Hid phenotype system: +> 2-fold of N2, ++> 10-fold of df only, (+)> 2-fold pdf only and (++)> 10-fold pdf only. Here, Hid is defined primarily by increased % df, but where no difference is seen, increased % pdf is considered. This gives a severity score ranking of ++ > + > [++] > [+].

Strain	% df	% pdf	% df/pdf *vs.* wildtype	% df/pdf *vs.* NL3908	% df/pdf *vs.* out-crossed	n
Wildtype	[1] 2.85	0				35
	[2] 5.71	0				70
	[3] 0	18.1				116
	[4] 0	0				139
	[5] 0	1.6				126
	[6] 3.12	16.7				96
	[7] 0	9.2				87
TJ1052	[1] 82.81	0	++			64
	[2] 27.33	0	++			139
	[3] 78.08	0	++			73
	[4] 98.48	0	++			66
	[5] 100	0	++			89
	[6] 92.23	0	++			103
	[7] 68.75	4.7	++			64
GR1307	[1] 0	0	-			35
	[2] 0	0	-			159
	[4] 0	0	-			96
	[5] 0	0	-			106
	[6] 0	0	-			107
	[7] 0	0	-			100
DP38	[2] 0	0	-			26
	[3] 0	0	-			24
	[4] 0	0	-			24
	[5] 0	8.9	[+]			45
	[6] 0	23	-			126
	[7] 0	27.3	[+]			22
NL3909	[1] 5.88	0	+	++		34
	[2] 4.65	0	-	++		86
	[3] 0	84.3	[+]	[+]		70
	[4] 6.93	53.5	++	+		101
	[5] 20.93	65.1	++	++	++	86
	[6] 28.98	68.1	+	[+]	+	69
	[7] 0	62.5	[+]	-	[++]	16
NL3908	[1] 0	0	-			33
	[2] 0	0	-			39
	[3] 8	44	++			25
	[4] 2.72	71.8	++			110
	[5] 0	86.2	[++]		[+]	58
	[6] 40.42	38.3	++		++	47
	[7] 0	60	[+]		[++]	10
GA905	[5] 0	0	-			138
	[6] 3.96	50.5	[+]			101
	[7] 0	5.1	-			136
GA906	[5] 0	0	-			199
	[6] 0	9.4	-			106
	[7] 0	2	-			153

Given the level of variation between individual trials, it was not possible to do statistical analysis. Therefore each trial is presented separately in Table 6.5. Overall, both of the original transgenic lines, NL3909 and NL3908, have a higher dauer formation frequency than wildtype animals (trial 3, 4, 5, 6, 7) i.e. are Hid. This is consistent with the presence of *daf-?*. By contrast, the out-crossed derivatives GA905 and GA906 are not Hid, presumably because *daf-?* has been removed by the backcrossing. Thus it is possible that the *daf-?* is the real cause of the extended life span in NL3909, and to a lesser extent in NL3908.

The strain with the reported *daf-?* mutation, DP38 was not Hid, presumably because this trait is suppressed by the *daf-d* mutation *unc-119(ed3)*.

6.2.6. Is *daf-?* mutation in DP38 sufficient to increase life span in NL3909?

To test this I measured the life span of three other DP38 transformant strains. I. Hope from the University of Leeds kindly provided the strains, UL2437 *lels2437*, UL2451 *lels2451* and UL2750 *lels2750*. These strains were generated by microbombarding the *unc-119(ed3) III; daf-?* strain DP38 with a plasmid carrying the wild type *C. elegans unc-119* gene, rescuing the Unc phenotype. The life span experiments were performed at 20˚C, using 40 µM FUdR to prevent progeny production (see Materials & Methods, section 2.2.4.). The results are presented in Figure 6.7 and Table 6.6.

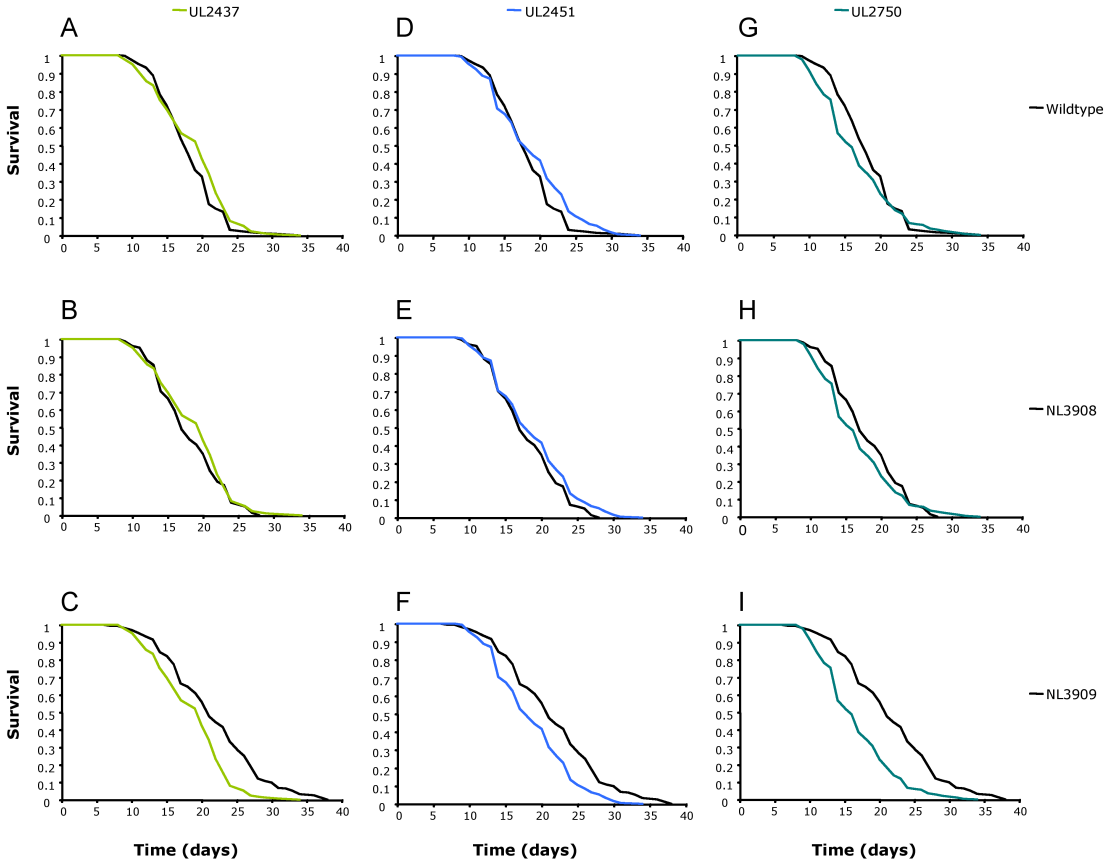


Figure 6.7: Life span of NL3909 background strains. Genotypes: N2 wildtype, NL3909 *unc-119(ed3) III (pkIs1642[unc-119(+); sir-2.1(+)])*, NL3908 *unc-119(ed3) III (pkIs1641[unc-119(+)])*, UL2437 *unc-119(ed3)III* (*lels2437*), UL2451 *unc-119(ed3)III* (*lels2451)* and UL2750 *unc-119(ed3)III* (*lels2750).* **(A-I)** Life span curves of the three transformants compared with wildtype, NL3908 and NL3909. The life span curves represent the combined data of at three independent biological replicates. The statistical analysis is presented in Table 6.6.

Table 6.6: Life span of NL3909 background strains. The trials were performed at 20°C with 40µM FUdR. Genotypes: N2 wildtype, NL3909 *unc-119(ed3) III (pkIs1642 [unc-119(+); sir-2.1(+)])*, NL3908 *unc-119(ed3) III (pkIs1641 [unc-119(+)])*, UL2437 *unc-119(ed3) III* (*lels2437)*, UL2451 *unc-119(ed3) III* (*lels2451)* and UL2750 *unc-119(ed3) III* (*lels2750). p*, log rank test. [n] biological replicate number.

Strain	Deaths/ censored	Mean life span (days)	% *vs.* wildtype	*p vs.* wildtype	% *vs.* NL3908	*p vs.* NL3908	% *vs.* NL3909	*p vs.* NL3909
Wildtype	**[C] 254/115**	**18.2**						
	[1] 58/68	17.1						
	[2] 90/28	17.4						
	[3] 106/19	19.4						
NL3909	**[C] 341/33**	**21.7**	**+19.2**	**<.0001**	**+19.9**	**<.0001**		
	[1] 114/13	21.9	+28.1	<.0001	+10.1	0.0004		
	[2] 117/11	21.5	+23.6	<.0001	+24.3	<.0001		
	[3] 110/9	21.8	+12.4	0.0006	+27.5	<.0001		
NL3908	**[C] 330/48**	**18.1**	**-0.5**	**0.30**			**-16.6**	**<.0001**
	[1] 113/19	19.9	+16.4	<.0001			-9.1	0.0004
	[2] 118/10	17.3	-0.6	0.89			-19.5	<.0001
	[3] 99/19	17.1	-11.9	0.003			-21.6	<.0001
UL2437	**[C] 189/65**	**18.9**	**+3.8**	**0.02**	**+4.4**	**0.04**	**-12.9**	**<.0001**
	[1] 85/34	20.4	+19.3	<.0001	+2.5	0.57	-6.8	0.005
	[3] 104/31	17.6	-9.3	0.04	+2.9	0.36	-19.3	<.0001
UL2451	**[C] 307/72**	**18.8**	**+3.3**	**0.02**	**+3.9**	**0.009**	**-13.4**	**<.0001**
	[1] 69/51	21.7	+26.9	<.0001	+9	0.002	-0.9	0.84
	[2] 115/12	18.3	+5.2	0.07	+5.8	0.05	-14.9	<.0001
	[3] 123/9	17.5	-9.8	0.01	+2.3	0.42	-19.7	<.0001
UL2750	**[C] 322/50**	**16.9**	**-7.1**	**0.004**	**-6.6**	**0.007**	**-22.1**	**<.0001**
	[1] 97/29	19.5	+14	0.0003	-2	0.57	-11	0.0002
	[2] 116/8	15.7	-9.8	0.007	-9.2	0.02	-27	<.0001
	[3] 109/13	15.8	-18.6	<.0001	-7.6	0.05	-27.5	<.0001

Two of the transformant strains (UL2437 and UL2451) were slightly but significantly longer lived than wildtype (p=0.02 in each case), but significantly shorter-lived than NL3909 (p<.0001). UL2750 was shorter-lived than wildtype animals (p<.0001). This means that it is unlikely that *daf-?* accounts for the longevity of NL3909.

These trials demonstrate the importance of several biological replicates. The first trial showed no significant difference in life span between UL2451 and NL3909 (p=0.84), supporting the theory that the *daf-?* genetic background rather than the *sir-2.1* transgene causes the extended life span of the NL3909 *sir-2.1* over-expresser line. However, two further trials did not replicate this finding (Table 6.6). In conclusion, NL3908 and NL3909 contain a second site mutation that makes them Hid. This they may have inherited from the parental strain DP38. However DP38 does not contain a background mutation that explains NL3909 longevity. The cause of NL3909 longevity remains unknown.

6.3. Discussion

6.3.1. The sirtuin controversy

Given that sirtuins have featured so prominently in ageing research during the recent years, it is important to investigate if and how exactly these proteins are involved in ageing. The idea that sirtuins increase life span originated with the discovery that *SIR2* over-expression in yeast results in increased replicative life span (Kaeberlein et al., 1999). Since sirtuins are conserved across many phylums, it was thought they might have the same effect in other species. However, the mechanism through which *SIR2* increases life span in yeast (suppression of ERC formation) is specific to yeast, and therefore cannot be operative in higher eukaryotic organisms. It was therefore somewhat unexpected that over-expression of genes homologous to yeast *SIR2* homologues gene resulted in extended life span in *C. elegans* and *Drosophila* too (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). The effects of the putative sirtuin activator resveratrol on life span in yeast, *C. elegans* and *Drosophila* were also taken as evidence for the role of sirtuins in longevity (see Introduction, section 1.6.4.) (Howitz et al., 2003; Wood et al., 2004).

However, more recent studies have shown that resveratrol does not in fact activate sirtuins (Beher et al., 2009; Pacholec et al., 2010). Moreover, the effect of resveratrol on life span in *Drosophila* was not reproducible and only in *C. elegans* the small effects in life span seen were not *sir-2.1* dependent (Bass et al., 2007). The appearance of articles such as “Resveratrol is dead, long live resveratrol” in Nature (Gallaway, 2010) and the halt to production of

the Sirtris resveratrol formulation SRT501 (Staff, 2010), highlight the controversy of this topic.

6.3.2. Over-expression of *sir-2.1* does not increase *C. elegans* life span

Evidence that *sir-2.1* over-expression leds to an increase in life span in *C. elegans* is derived in particular from two *sir-2.1* over-expressing strains, LG100 and NL3909 (Berdichevsky et al., 2006; Tissenbaum and Guarente, 2001; Viswanathan et al., 2005). Surprisingly, the data presented here on both over-expressing strains shows that the increased life span is caused by genetic background effects rather than the elevated *sir-2.1* levels. Standardizing the genetic background abolished the life span extension in both cases.

The life span extension in LG100 is caused by a second site mutation resulting in a Dyf phenotype (see Chapter 5). We tested both NL3908 and NL3908 for the Dyf phenotype but this was not present. So far the reason for the increased life span in NL3909 is unclear. Tests on three independent DP38-derived transformants excluded the possibility that the cause was a factor inherited from this parent strain.

sir-2.1 RNAi did not reduce NL3909 life span. However, on *E. coli* HT115, NL3909 was not longer lived than NL3908, apparently due to increased NL3908 longevity on this *E. coli* strain. The extended life span of LG100 was also reduced on the RNAi bacteria HT115 (mean of 25.28 days on OP50 *vs.* 20.93 days on HT115). This makes sense, since the second site *dyf* mutation in this strain probably affects the cilia in the sensory neurons,

which can mediate food-source dependent effects on life span (Maier et al., 2010) (see Chapter 5). Food and sensory neurons have both been shown to mediate extensions in life span via DR. Thus LG100 might be long-lived because of food limitation caused by its impaired ability to sense food. However, the effects of differences in food-source on life span are distinct from those of DR. For instance, DR treated worms show a decrease in development and fecundity (Klass, 1977), which are not affected in LG100. This suggests that food-source and DR effects on *C. elegans* life span do not involve identical mechanisms (Maier et al., 2010). However, it remains possible that DR effects are at least partially mediated by food perception effects.

The extension of life span in NL3909 is not just reduced on HT115 bacteria, as in LG100, but no longer significant relative to its control strain NL3908 (p=0.07). Here, our findings are different to those previously reported (Berdichevsky et al., 2006). In the previous study, NL3909 was not only long-lived on HT115 bacteria (mean of 32 days) compared to NL3908 (mean of 24 days), but this increase in life span was also *sir-2.1*-dependent (mean of 24 days on *sir-2.1* RNAi) (Berdichevsky et al., 2006). The conflicting results might reflect laboratory differences in culture conditions. Animals of the same strain do sometimes show differences in life span in different laboratories (Partridge and Gems, 2007). In our trials, worms were kept for two generations on the RNAi cultures prior the start of the life span, whereas the life span in the prior study were initiated by transferring L4 animals to the corresponding RNAi plates. Keeping worms for several generations on RNAi bacteria increases rather than reduces the RNAi effect. Therefore this methodological difference

seems unlikely to account for the discrepant results. To exclude the possibility that FUdR used in the prior study might be the reason for the discrepancy, we included one trial with 10 µM FUdR (see Table 6.4, trial [4]). However, the results were the same with FUdR as in the three previous trials without FUdR. As previously mentioned, it is unclear how many RNAi trials were performed in the previous study (Berdichevsky et al., 2006), supplemental Table S2 A). If only a single trial was performed, it seems possible that the observed *sir-2.1* RNAi effect on NL3909 life span is artefactual. If we consider the RNAi data presented in this thesis, trial [1] shows a significant decrease in life span of NL3909 on *sir-2.1* RNAi compared to control animals (p=0.0002), which confirms the finding in the earlier study. However, trial [2] shows no difference between NL3909 animals on control RNAi compared to *sir-2.1* (p=0.42) and two more trials ([3] and [4]) show a significant increase in life span on *sir-2.1* RNAi plates (p<.0001/0.001). This underscores the importance in life span studies of performing multiple replicate trials.

The loss of the NL3909 longevity on HT115 in our trials was caused partly by a 4.77% decrease in life span (mean of 21.54 days on HT115 *vs.* 22.62 days on OP50), and partly by a 8.98% increase in life span of NL3908 (mean of 20.87 days on HT115 *vs.* 19.15 days on OP50). This opposite reaction in the two strains is difficult to reconcile with the view that both strains have the same genetic background, apart from the additional copies of *sir-2.1* in NL3909. This strain difference might be caused by a spontaneous mutation in one of them, causing the divergent food-source dependent effect in life span.

One possibility is that the *daf-?* mutation inherited from DP38 causes an increase in life span that is *E. coli* strain dependent, i. e. seen with HT115 but not OP50. NL3909 might have a second mutation that makes it resistant to the life shortening effect of OP50. This would predict that the three DP38 transformants from the Hope lab would be long-lived on HT115 too.

Another possibility raised by my RNAi results is that *sir-2.1* has a negative effect on life span. Four different strains with different genetic backgrounds are consistently longer-lived on *sir-2.1* RNAi bacteria (GA707 mean of 18.5 days on L4440 *vs.* 19.4 days on *sir-2.1* RNAi, p=0.006 [see Chapter 5, N2 mean of 18.3 days on L4440 *vs.* 18.97 days on *sir-2.1* RNAi, p=0.05, NL3908 mean of 20.9 days on L4440 *vs.* 22 days on *sir-2.1* RNAi, p=0.001 and NL3909 mean of 21.5 on L4440 *vs.* 22.5 days on *sir-2.1* RNAi, p=0.001 [see Figure 6.6, Table 6.4]). Even LG100 shows a 4.7% increase on *sir-2.1* RNAi compared to L4440, but the difference does not quite reach significance (p=0.06).

Could *sir-2.1* really shorten *C. elegans* life span? Arguing against this is that the out-crossed derivative of LG100, GA468, over-expresses *sir-2.1*, but is not short-lived compared to wildtype (p=0.90) or *rol-6* control animals (p=0.82). One interpretation is that reducing SIR-2.1 levels can increase life span, but increasing SIR-2.1 levels does not decrease it. Notably, deletion of *sir-2.1* markedly increases life span in a *daf-2* mutant background (Berdichevsky et al., 2006; Burnett et al., 2011).

6.3.3. Perspectives of the sirtuin affair

This study is not the only recent example in biogerontology showing that poorly controlled genetic background can led to erroneous conclusions. It was reported that mutation of the gene *Indy (I'm not dead yet)*, previously thought to increase life span in *Drosophila* (Rogina et al., 2000), has no effect on ageing after correction for background effects (Toivonen et al., 2007). *Indy* encodes a *Drosophila* Krebs cycle intermediate transporter that was thought to mimic DR effects. Similarly, a new study shows that *dSir2* over-expression does not increase life span in *Drosophila* when appropriate transgenic controls are included (Burnett et al., 2011). This contradicts the conclusion of an earlier study (Rogina et al., 2000), which did not include these controls.

There is no doubt that sirtuins are an important and interesting class of proteins, yet their importance in ageing appears questionable (apart from in replicative ageing in budding yeast). However, it is of course possible that *sir-2.1* has effects on ageing in different contexts and conditions to those used in this study. Although *sir-2.1* may not seem to play a major role in *C. elegans* life span, the *geIn3* transgene of the LG100 strain rescued early neuronal dysfunction phenotypes in a *C. elegans* model for Huntington's disease (Parker et al., 2005). It was subsequently confirmed that this neuroprotective effect is not caused by the Dyf mutation (Burnett et al., 2011). Moreover, NL3909 is thermotolerant (Burnett et al., 2011) and this effect is *sir-2.1* dependent (Berdichevsky et al., 2006). Thus, *sir-2.1* over-expression clearly affects *C. elegans* metabolism.

The data presented in this thesis contradicts the view that sirtuins protect against ageing in *C. elegans*. The longevity of the two *sir-2.1* over-

expressing strains tested is not *sir-2.1*-dependent, and disappears after standardization of the genetic background. Taking together my findings and other recent work challenging the sirtuin-ageing theory, it seems questionable whether sirtuins are really the “Holy Grail” against ageing that they have been thought to be for a number of years.

Conclusion

Science is the Latin word for knowledge and essentially describes measurable and predictable explanations about natural properties and functions. In the past science was closely linked to philosophy. Nowadays science is more based on measurable data and facts. Naturally, throughout human history people have tried to explain various natural conditions with different outcomes. Many theories were postulated and again overthrown by newer findings or ideas. For instance, for centuries it was believed that the world was at the centre of the universe until Copernicus, Kepler and Galilei put them on the right track, from the geocentric to the heliocentric model.

Both of my projects have investigated the truthfulness of widely held beliefs in biogerontology. The oxidative damage theory of ageing dominated the ageing field for over five decades, while the proposed role of sirtuins in ageing has been influential during the last decade. Meanwhile, numerous studies, including my work presented here, question the very core of both conceptions. These controversies show how important careful testing of a hypothesis from different angles is. In the case of the oxidative damage theory, there is a lot of data supporting its view, but also a lot contradicting it. The view of sirtuins as anti-ageing genes started in yeast and was then extended to other organisms. Yet now the role of sirtuins in animal ageing is very uncertain.

Biogerontology is a very complicated area of science: ageing is a very complex process and its primary causes are still unknown. Many factors can influence the life span of organisms, such as culture conditions, genetic

background or maternal effects. Considering how complex organisms are, from bacteria up to mammals, it is risky to exclude or apply standing theories to all of them, i.e. to over-generalize. It could well be that both ROS and sirtuins play major roles in ageing in some organisms in ways not yet tested, but not in others. However, contradictory data for both theories have been reported in invertebrates as well as vertebrates. This demonstrates the importance of testing a theory by its reproducibility in different labs using different techniques and organisms.

Socrates once said, “The more you know, the more you realize you know nothing”. This seems particularly fitting to the current status of our knowledge of the biology of ageing. Although our understanding of ageing is growing that does not mean that the foundations of this knowledge are untouchable or complete. Not to challenge them risks arriving at wrong conclusions and misinterpretations. Although correct challenges often give rise to new questions rather than answers these question at least set us on the right path to truth.

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LETTER

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Absence of effects of Sir2 overexpression on lifespan in *C. elegans* and *Drosophila*

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Overexpression of sirtuins (NAD^+-dependent protein deacetylases) has been reported to increase lifespan in budding yeast (*Saccharomyces cerevisiae*), *Caenorhabditis elegans* and *Drosophila melanogaster*[1–3]. Studies of the effects of genes on ageing are vulnerable to confounding effects of genetic background[4]. Here we re-examined the reported effects of sirtuin overexpression on ageing and found that standardization of genetic background and the use of appropriate controls abolished the apparent effects in both *C. elegans* and *Drosophila*. In *C. elegans*, outcrossing of a line with high-level *sir-2.1* overexpression[1] abrogated the longevity increase, but did not abrogate *sir-2.1* overexpression. Instead, longevity co-segregated with a second-site mutation affecting sensory neurons. Outcrossing of a line with low-copy-number *sir-2.1* overexpression[2] also abrogated longevity. A *Drosophila* strain with ubiquitous overexpression of *dSir2* using the UAS-GAL4 system was long-lived relative to wild-type controls, as previously reported[3], but was not long-lived relative to the appropriate transgenic controls, and nor was a new line with stronger overexpression of *dSir2*. These findings underscore the importance of controlling for genetic background and for the mutagenic effects of transgene insertions in studies of genetic effects on lifespan. The life-extending effect of dietary restriction on ageing in *Drosophila* has also been reported to be *dSir2* dependent[3]. We found that dietary restriction increased fly lifespan independently of *dSir2*. Our findings do not rule out a role for sirtuins in determination of metazoan lifespan, but they do cast doubt on the robustness of the previously reported effects of sirtuins on lifespan in *C. elegans* and *Drosophila*.

The role of sirtuins in ageing was discovered in budding yeast, where overexpression of *SIR2* increases replicative lifespan[5]. It was then reported that elevated sirtuin levels increase lifespan in the nematode *C. elegans*[1,2,6] and the fruitfly *Drosophila*[3], indicating an evolutionarily ancient role of sirtuins in longevity assurance[7]. Dietary restriction (reduced food intake short of starvation) extends lifespan in organisms ranging from yeast to mammals[8], and initial studies indicated that dietary restriction increases lifespan by activating sirtuins in yeast[9], *C. elegans*[10] and *Drosophila*[3]. Pharmacological activation of sirtuins has therefore been widely promulgated as a potential means to mimic dietary restriction and slow ageing in humans[11]. However, several aspects of the role of sirtuins in ageing have proved controversial[12]. Subsequent studies have indicated that sirtuins do not mediate the effects of dietary restriction on ageing, at least in budding yeast and *C. elegans*[13,14]. The plant-derived polyphenol resveratrol and other compounds have been reported to activate sirtuins and extend lifespan[15,16], but more recent findings have challenged both effects[17–20]. We therefore re-examined the effects of sirtuin overexpression on lifespan in *C. elegans* and *Drosophila*. In particular, we wished to exclude the possibility that the increased longevity observed in strains with overexpression of sirtuin genes is caused by differences in genetic background, or by the mutagenic effects of transgene insertion, which frequently confound studies of the genetics of ageing[4].

We first examined a high-copy-number *sir-2.1* transgenic *C. elegans* strain (LG100) carrying the integrated transgene array *geIn3* [*sir-2.1 rol-6*(*su1006*)] (ref. 1). As expected, this strain was long-lived (Fig. 1a and Supplementary Table 1). However, outcrossing (×5) of *geIn3* to wild type (N2) abrogated the increase in longevity (Fig. 1a and Supplementary Table 1) without affecting SIR-2.1 protein levels (Fig. 1b). This loss of longevity upon outcrossing was verified by an independent research team (Supplementary Table 2).

LG100 showed a neuronal dye-filling (Dyf) defect[21] that did not segregate with the transgene upon outcrossing (Supplementary Fig. 2a). Dyf mutants often show extended lifespan[22]. To determine whether the longevity of LG100 might be attributable to a *dyf* mutation, we derived from this strain three Dyf, non-Rol lines (lacking *geIn3*) and three non-Dyf, Rol lines (carrying *geIn3*). Dyf, non-Rol lines were long-lived and showed wild-type SIR-2.1 protein levels (Fig. 1c, d and Supplementary Table 3). Non-Dyf, Rol lines showed elevated SIR-2.1 protein levels but had wild-type lifespans. Dyf mutant longevity seemed to be partially dependent on *daf-16* (Supplementary Fig. 2b), as seen previously for other Dyf mutants[22]. The co-segregation of longevity with this *dyf* mutation, but not with *geIn3*, was previously noted by another research team (S. S. Lee, personal communication). Furthermore, knockdown of *sir-2.1* expression in LG100 using RNA-mediated interference did not suppress longevity, despite lowering SIR-2.1 protein to wild-type levels (Fig. 1e, f and Supplementary Table 4). Taken together, these results indicate that the longevity of LG100 is attributable to an unidentified *dyf* mutation (or possibly another mutation closely linked to the *dyf* locus), and that high-level overexpression of *sir-2.1* is not sufficient to increase lifespan in these strains.

A low-copy-number transgenic strain (NL3909) overexpressing *sir-2.1* (ref. 7) is also long-lived[2]. We confirmed the increased lifespan of NL3909 (*pkIs1642 [sir-2.1 unc-119] unc-119*(*ed3*)) relative to the control strain NL3908 (*pkIs1641 [unc-119] unc-119*(*ed3*)) (Fig. 1g and Supplementary Table 5). We also observed an apparent increase in SIR-2.1 protein levels in NL3909 relative to NL3908 (Fig. 1h). Outcrossing (×6) of NL3909 once again abrogated longevity (Fig. 1g and Supplementary Table 5) without affecting SIR-2.1 protein levels (Fig. 1h and Supplementary Fig. 1c). This effect of outcrossing was independently verified (Supplementary Table 6). Thus, the longevity of NL3909 also seems to be attributable to effects of genetic background rather than to *pkIs1642*.

The duplication *mDp4* includes the *sir-2.1* locus, and the *mDp4*-containing strain DR1786 is long-lived[1]. We found that DR1786 is

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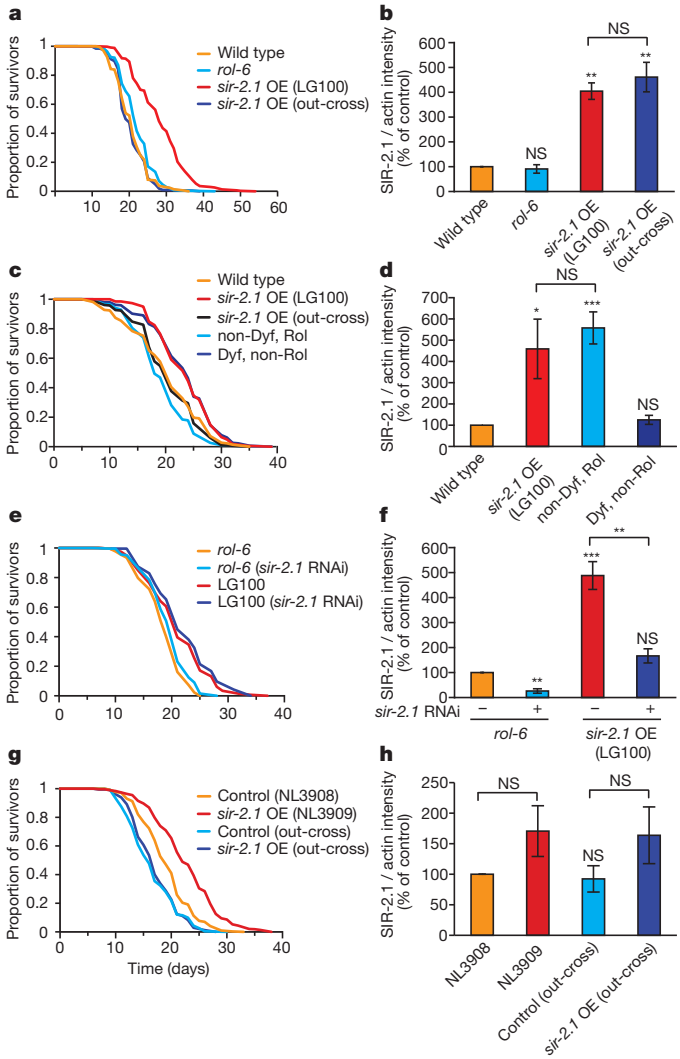

Figure 1 | Longevity of LG100 and NL3909 is not attributable to *sir-2.1* overexpression in *C. elegans*. **a**, **b**, Outcrossing of LG100 removes lifespan extension without affecting SIR-2.1 protein levels. Data in **b** are derived from western blots (mean of three trials, each using an independent protein preparation). A representative western blot is shown in Supplementary Fig. 1a. Quantitative reverse transcriptase PCR showed that *sir-2.1* mRNA is also elevated in both strains (data not shown). **c**, LG100-derived Dyf, non-Rol segregant lines are long-lived whereas non-Dyf, Rol lines are not. **d**, Non-Dyf Rol segregant lines have elevated SIR-2.1 levels, whereas Dyf, non-Rol lines do not. **e**, **f**, *sir-2.1* RNAi does not suppress LG100 longevity, but reduces SIR-2 protein levels. **g**, **h**, Outcrossing of NL3909 removes lifespan extension without affecting SIR-2.1 protein levels. See Supplementary Tables 1–5 for lifespan statistics for **a**, **c**, **e** and **g**, respectively. OE, overexpression. All error bars represent s.e.m. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$; NS, not significant; Student's *t*-test (two-tailed). One remaining possibility is that the outcrossed *sir-2.1* strains both contain second-site mutations that suppress longevity effects. However, *daf-2* RNAi strongly induced longevity in both strains (data not shown), arguing against the presence of a general suppressor of longevity in each case.

indeed long-lived, and also shows elevated *sir-2.1* expression. However, longevity was not suppressed by *sir-2.1* RNA interference (RNAi) (Supplementary Fig. 3 and Supplementary Table 7) indicating causation by factors other than *sir-2.1*, either on *mDp4* or elsewhere in the genome.

In *Drosophila*, overexpression of *dSir2* reportedly increases lifespan relative to wild-type controls[3]. Overexpression was achieved using the GAL4-UAS binary system[23], with the largest increases in lifespan being produced by the combination of EP-UAS–*dSir2* ($dSir2^{EP2300}$) with a ubiquitously expressed tubulin–GAL4 driver. We outcrossed these

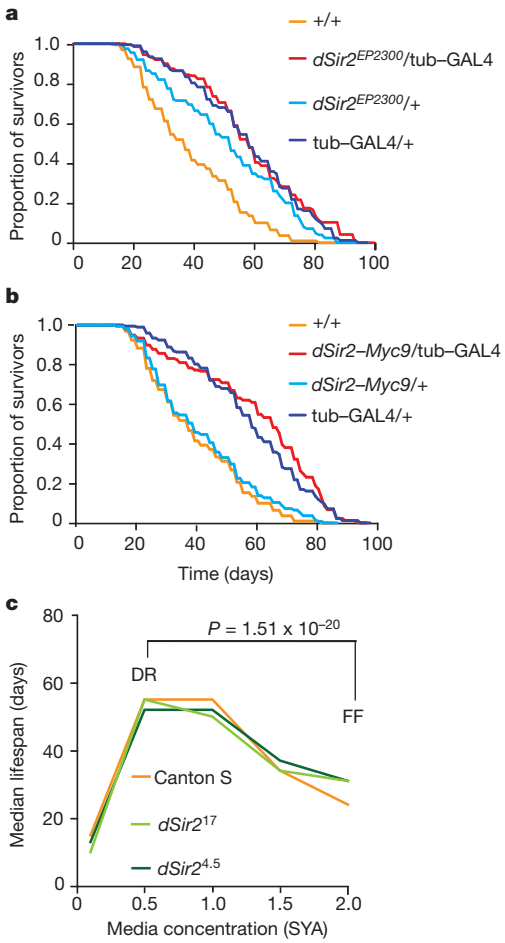

Figure 2 | Absence of effects of *dSir2* on lifespan in *Drosophila*. All lines were outcrossed into w^{Dah} (+/+). **a**, Lifespan in flies overexpressing $dSir2^{EP2300}$ driven via tubulin–GAL4 (tub–GAL4) is longer than in the wild type, but not longer than in the *tubulin–GAL4*/+ genetic control. Median lifespans: +/+, 39 days; $dSir2^{EP2300}$/*tubulin–GAL4*, 59 days; $dSir2^{EP2300}$/+, 53 days, *tubulin–GAL4*/+, 60 days. $P = 0.0006$ for comparison of $dSir2^{EP2300}$/*tubulin–GAL4* versus $dSir2^{EP2300}$/+; $P = 0.9295$ for $dSir2^{EP2300}$/*tubulin–GAL4* versus *tubulin–GAL4*/+; $P < 0.0001$ for $dSir2^{EP2300}$/*tubulin–GAL4* versus +/+. **b**, Lifespan in flies overexpressing *dSir2–Myc9* is longer than in wild type, but not longer than in the *tubulin–GAL4* control. Median lifespans: +/+, 39 days; *dSir2–Myc9*/*tubulin–GAL4*, 67 days; *dSir2–Myc9*/+, 41 days; *tubulin–GAL4*/+, 60 days. *dSir2–Myc9*/*tubulin–GAL4* versus *dSir2–Myc9*/+, $P = 0.0001$; *dSir2–Myc9*/*tubulin–GAL4* versus *tubulin–GAL4*/+, $P = 0.1354$; *dSir2–Myc9*/*tubulin–GAL4* versus +/+, $P < 0.0001$. All comparisons were made using log-rank tests, $n = 200$. **c**, The effect of dietary restriction on *Drosophila* lifespan is not *dSir2*-dependent. Flies were assayed over five concentrations of SYA media and data are presented as the median lifespan on each food concentration. All lines were outcrossed into Canton S (+/+). *P* values confirm that all flies respond normally to dietary restriction when median lifespans are compared for dietary restriction (DR) versus fully-fed (FF) conditions[30].

two transgenes (×6) into the control white Dahomey (w^{Dah}) background. When assayed on a medium similar to that used in the original study, EP-UAS–*dSir2*/*tubulin–GAL4* flies were longer-lived than wild-type controls, as previously reported[3] (Fig. 2a). However, they did not live longer than the *tubulin–GAL4*/+ control flies (Fig. 2a). This implies that lifespan extension is due to transgene-linked genetic effects other than the overexpression of *dSir2*. Lifespan was assayed on a range of food media (see Methods for details) to test for nutrient dependence of any effect. However, in no case were EP-UAS–*dSir2*/*tubulin–GAL4* flies longer-lived than one or both transgenic controls (Supplementary Fig. 4).

The lack of an observable effect on lifespan could reflect the relatively modest increase in *dSir2* expression in EP-UAS–*dSir2*/*tubulin–GAL4*

flies, both in terms of messenger RNA levels (Supplementary Fig. 5) and protein levels (increased by 35% relative to wild type; Supplementary Fig. 6). We therefore created lines with a higher level of overexpression of *dSir2* (UAS–*dSir2–Myc9*/*tubulin–GAL4*). Here, *dSir2* mRNA and protein levels were robustly increased relative to wild type (an increase of 318% relative to wild-type protein levels; Supplementary Figs 5 and 6). We examined recombinant protein raised in *Escherichia coli* to check that the presence of the Myc tag did not interfere with dSir2 histone deacetylase activity, as measured by deacetylation of the fluorophore-containing p53 substrate (Fluor de Lys) or of native acetylated histone H4 substrates, and it did not (Supplementary Fig. 7). We also found that dSir2 histone deacetylase activity was unaffected by addition of resveratrol in either assay (Supplementary Fig. 7). We saw no increase in lifespan in UAS–*dSir2–Myc*/*tubulin–GAL4* flies relative to *tubulin–GAL4*/+ controls, either on a food medium similar to that used in the original study (Fig. 2b), or relative to either control on a range of other media (Supplementary Fig. 4b, c, f). An independent research team also saw no increase in lifespan in UAS–*dSir2–Myc9*/*tubulin–GAL4* flies (Supplementary Fig. 8). These results indicate that the previously observed longevity of EP-UAS–*dSir2*/*tubulin–GAL4* flies was not attributable to elevated expression of *dSir2*, and that stronger, ubiquitous overexpression of *dSir2* also does not extend fly lifespan.

The role of sirtuins in the extension of lifespan by dietary restriction in yeast and *C. elegans* is controversial, with several groups reporting that sirtuins are not required for lifespan extension via dietary restriction in both organisms[8]. In *Drosophila*, it was reported that dietary restriction does not increase lifespan in *dSir2* deletion-mutant flies[3]. We tested this too, using the deletion alleles $dSir2^{4.5}$ (tested previously[3]) and $dSir2^{17}$. We first outcrossed these alleles (Supplementary Fig. 9a) into the Canton S wild type (see Methods), which was used in the previous dietary-restriction study[3]. We then checked the effect of each allele on *dSir2* gene expression. The allele $dSir2^{17}$ abrogated *dSir2* mRNA, indicating that this is a null allele. By contrast, $dSir2^{4.5}$, which contains a relatively small deletion at the 5′ end of the gene, did not reduce *dSir2* mRNA levels (Supplementary Fig. 9b, c).

To reassess the role of *dSir2* in dietary restriction in *Drosophila*, we compared lifespans of wild-type (Canton S), $dSir2^{4.5}$ and $dSir2^{17}$ homozygotes. All genotypes responded similarly and normally to dietary restriction in trials conducted by two independent research teams (Fig. 2c and Supplementary Fig. 10), hence the effect of dietary restriction on lifespan did not require *dSir2*.

In this study, we were unable to verify the effect of sirtuin overexpression on lifespan in either *C. elegans* or *Drosophila*. Increased lifespan was seen in two *C. elegans* lines with elevated *sir-2.1* expression, derived from independent studies, as previously reported, but in each case this was abrogated by outcrossing. Overexpression of *sir-2.1* does exert effects on traits other than lifespan. For example, *geIn3* is neuroprotective in a worm model of neuron dysfunction in Huntington's disease[24] and, notably, this effect is not attributable to the *dyf* mutation (Supplementary Fig. 11). Moreover, both NL3909 and its outcrossed derivative are thermotolerant (M. Somogyvári and C. Sőti, unpublished data). In *Drosophila*, lines overexpressing *dSir2* were longer-lived than wild-type controls, as previously reported, but they were not longer-lived than lines containing the appropriate transgenic controls. The fact that all transgenic lines were longer-lived than the Dahomey wild type into which they had been outcrossed could reflect heterosis in the vicinity of the transgene inserts, or a mutagenic effect of the GAL4 insert.

Lifespan was not increased either by overexpression of *sir-2.1* from its own promoter in *C. elegans,* or by ubiquitous overexpression of *dSir2* from a heterologous promoter in *Drosophila*. Our findings call into question the robustness of earlier reports of a role for sirtuins in longevity assurance on the basis of overexpression in *C. elegans* and *Drosophila*, and also the role of *dSir2* in the response to dietary restriction in *Drosophila*. However, sirtuins can affect lifespan in animals under certain conditions: *C. elegans daf-2(e1370)* mutants are hypersensitive to genetic effects on lifespan[25], and in these mutants, deletion of *sir-2.1* reproducibly increases lifespan[6] (Supplementary Fig. 12).

Our finding that resveratrol does not activate the histone deacetylase activity of dSir2 using a native histone H4 peptide is consistent with earlier findings using yeast SIR2 and mammalian SirT1 (refs 17, 18). Resveratrol increased *Drosophila* lifespan in one study[26] but not in another[27]. In principle, this could reflect sensitivity of resveratrol effects to subtle differences in culture conditions. If this were the case, our findings would indicate that such effects are not attributable to direct activation of dSir2 by resveratrol.

METHODS SUMMARY

Nematode strains and maintenance. Nematodes were maintained on nematode-growth-medium agar at 20 °C, with *E. coli* OP50 bacteria as a food source. Nematode strains used included: wild type (N2), GA707 *wuEx166 [rol-6(su1006)]* (*rol-6* control), LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*, NL3909 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)* and the control strain NL3908 *pkIs1641 [unc-119] unc-119(ed3)*.

Nematode lifespan measurements. These were performed as previously described[28], at 20 °C. To prevent progeny production, 5-fluoro-2′-deoxyuridine (FUdR) was added to seeded plates, to a final concentration of 10, 40 or 50 μM. Before testing the effects of RNAi on lifespan, worms were kept for two generations on the RNAi bacteria. The statistical significance of effects on lifespan was estimated using the log-rank test, performed using JMP, Version 7 (SAS Institute).

***Drosophila* stocks and maintenance.** *Tubulin–GAL4* and $dSir2^{EP2300}$ were obtained from the Bloomington Stock Center. The *dSir2–Myc2* and *dSir2–Myc9* lines were generated by germline transformation into strain w^{04}. The $dSir2^{4.5}$/SM6B, $dSir2^{17}$/Cyo and Canton S lines were gifts from S. Pletcher, J. Rine and S. Helfand. All lines were outcrossed at least six times into the relevant controls. Experiments were performed at 25 °C on a 12 h:12 h light:dark cycle at constant humidity.

***Drosophila* lifespan assays.** Flies were bred at standard density, allowed to mate for 48 h after emerging, then sorted into ten females per vial. Vials were changed every 48 h, and deaths per vial were scored until all flies were dead. In overexpression studies, $n = 200$. In *dSir2*-mutant studies, $n = 100$. For statistical methodology, see earlier.

dSir2 deacetylation assays. We used both the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences) and an HPLC-based acetyl-histone-H4 deacetylation assay[29]. *dSir2* and *dSir2–Myc* were cloned into pET SUMO (Invitrogen) and proteins were purified on HisPur cobalt spin columns (Thermo Scientific).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions The project was conceived by D.G. and L.P. and the experiments were designed by A.B., C.B., F.C., D.G., K.H., M.K., J.J.M., C.N., L.P., C.S. and S.V. The experiments were performed and analysed by C.A., D.A., C.B., F.C., J.J.M., M.G., M.H., A.-M.O., M.D.P., M.R., G.L.S., M.S., G.V., R.P.V.-M., S.V. and V.L. The manuscript was written by C.B., F.C., D.G., L.P. and S.V.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to D.G. (david.gems@ucl.ac.uk).

METHODS

Nematode strains and maintenance. *Caenorhabditis elegans* were cultured under standard monoxenic conditions[31,32]. Strains used included N2 (wild type), GA707 *wuEx166 [rol-6(su1006)]*, HT1593 *unc-119(ed3)*, LG100 *geIn3* [*sir-2.1 rol-6(su1006)*] *dyf-?(wu250)*, NL3908 *pkIs1641* [*unc-119*] *unc-119(ed3)* and NL3909 *pkIs1642* [*sir-2.1 unc-119*] *unc-119(ed3)*.

Outcrossing of nematode strains. LG100 was outcrossed with N2 and the Rol trait was used to detect the presence of *gIn3*. NL3908 and NL3909 were outcrossed using HT1593 *unc-119(ed3)*. Rescue of Unc (uncoordinated movement) was used to detect the presence of the transgene array.

Isolation of Dyf, non-Rol and non-Dyf, Rol lines. LG100 was crossed with N2 and lines were established from individual F_2 animals with Dyf, non-Rol or non-Dyf, Rol phenotypes. The Dyf phenotype was identified by staining with the dye 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) and looking for absence of dye uptake into the amphid and phasmid neurons. Non-Dyf, Rol F_2 animals that were heterozygous for the *geIn3* transgene array (the *rol-6* marker is dominant) were identified by the presence of non-Rol animals in the F_3, and were excluded.

RNAi in *C. elegans*. Animals were fed *E. coli* containing the HT115 vector, either with or without a portion of the *sir-2.1* gene cloned into it. The *sir-2.1* feeding strain was obtained from the Ahringer RNAi library[33]. Worms were maintained on RNAi feeding strains for two generations before lifespan measurements. One day before starting measurements, FUdR was applied to seeded plates at 10 μM to prevent progeny production.

Analysis of SIR-2.1 protein levels in *C. elegans*. Protein was prepared from synchronous nematode cultures (L4 larvae and young adults) raised on *E. coli* OP50 or RNAi bacteria for two generations. Western blots were performed with anti-actin monoclonal antibodies (Santa-Cruz Biotechnology) and an anti-SIR-2.1 polyclonal antibody (provided by A. Gartner[34]). For all assays, 3–5 replicate worm cultures were used.

Neuroprotection assays in *C. elegans*. To test for sirtuin-mediated protection from expanded polyglutamines (polyQs), we crossed GA919 (*geIn3* dissociated from *dyf-?(wu250)*) to strains carrying integrated polyQ arrays. These polyQ strains co-express the first 57 amino acids of human huntingtin with either 19 or 128 Gln residues fused to cyan fluorescent protein and expressed from the *mec-3* promoter, and YFP expressed from the *mec-7* promoter in touch-receptor neurons[24]. The response to touch at the tail was tested as previously described[24]. Three trials were performed and 150–200 animals were tested per genotype.

Lifespan analysis in *C. elegans*. Lifespans of synchronized population cohorts were measured as previously described[28]. FUdR was applied to the plates at 10, 40 or 50 μM (see Supplementary Tables). Lifespan experiments were performed at 20 °C. A small proportion of animals were censored, usually due to uterine rupture, which mainly occurred at mid-adulthood (~day 9–11).

Statistical analysis of *C. elegans* data. The statistical significance of effects on lifespan was estimated using the log-rank test, performed using JMP, Version 7 (SAS Institute).

***Drosophila* stocks and maintenance.** *Tubulin–GAL4* and $dSir2^{EP2300}$ lines were obtained from the Bloomington Stock Center. *dSir2–Myc2* and *dSir2–Myc9* lines were generated by germline transformation. These were outcrossed into white Dahomey (w^{Dah}). The strains $dSir2^{4.5}$/SM6B (ref. 35) and $dSir2^{17}$/Cyo (ref. 36), provided by S. Pletcher and J. Rine, were outcrossed into Canton S. All lines were outcrossed at least six times. The presence of the deletion was detected by PCR using the following primers: 149F (5′-AGATATGACATAAGGCAGTGGC-3′), 1427R (5′-TCCCGTTAGCACAATGATCTTC-3′) and 3909R (5′-GAAGGCGG TAGCAATGG TGACAA-3′). Flies were maintained at 25 °C on a 12 h:12 h light: dark cycle at constant humidity.

Myc-tagged *dSir2*. The Myc tag was added to RE27621 (Riken) using standard techniques and cloned into pUASP. The construct was microinjected into w^{04} and the transformant lines *dSir2–Myc2* and *dSir2–Myc9* were recovered. Primers were: Sir5′R2 (5′-CAAGAATTCCAACGAGAATTTTACACAGGTCGTGTG-3′), Sir3′Xba (5′-ATC GAGTCTAGACACTGCTGCTAACTGTCCTGGAGG-3′) and MYC3′Xba (5′-GAGCT ATCTAGAGGATCCGAGGAGCAGAAGCTGATC-3′).

Lifespan assays in *Drosophila*. Flies were bred at standard density (~300 flies per 200-ml bottle), allowed to mate for 48 h after emerging (once mated) and then sorted into ten females per vial (experiments performed at University College London) or 35 per vial on 15% SYA (experiments performed at University of Michigan). Vials were changed every 48 h and deaths per vial were scored until all flies were dead. The numbers of flies used in lifespan assays were: overexpression studies, $n \approx 200$ (UCL) or $n \approx 350$ (U. Michigan); dietary-restriction studies, $n = 100$. For the overexpression studies, the fly-food recipes were as follows: SYA (100 g yeast, 50 g sugar, 15 g agar, 30 ml nipagin and, in most trials, 3 ml propionic acid per litre of food); ASG (20 g yeast, 85 g sugar, 10 g agar and 60 g maize per litre of food); ASG^1 (31 g yeast, 124 g sugar, 9 g agar, 53 g cornmeal and 25 ml nipagin per litre of food); 15% SYA (150 g yeast, 150 g sugar, 21 g agar and 15 ml tegosept). For the dietary-restriction trials, the food dilutions used were as follows: 15 g agar, 30 ml nipagin, 3 ml propionic acid, with yeast and sugar both altered to final concentrations of 10 g, 50 g, 100 g, 150 g or 200 g per litre of food. All food was prepared as previously described[27].

Genetic crosses in *Drosophila*. *Tubulin–GAL4/TM3* males were crossed to $dSir2^{EP2300}$, *dSir2–Myc2* or *dSir2–Myc9* virgin females, and $dSir2^{EP2300}$/+; *tubulin–GAL4*/+, *dSir2–Myc2*/+; *tubulin–GAL4*/+ or *dSir2–Myc9*/+; and *tubulin–GAL4*/+ females were selected from the progeny. For the controls, *tubulin–GAL4/TM3* males or $dSir2^{EP2300}$, *dSir2–Myc2* or *dSir2–Myc9* virgin females were crossed to w^{Dah} and $dSir2^{EP2300}$/+, *tubulin–GAL4*/+, *dSir2–Myc2*/+ or *dSir2–Myc9*/+ females were selected from the progeny.

Quantitative reverse transcriptase PCR in *Drosophila*. RNA was extracted from ten females at 10 days of age using standard techniques and transcribed into cDNA. Four biological replicates were run per genotype, each in triplicate. Samples were normalized to either actin5C or ribosomal protein 49 (RP49). Primers used were: Sir2-4 5′-GCTCTCCACCGTTGTCTGAGGGCC-3′ (ref. 3), Sir2-5 5′-GGCGGCAGCTGTGCTGCGATGAG-3′ (ref. 3), Actin5CF 5′-CAC ACCAAATCTTACAAAATGTGTGA-3′, ActinCR 5′-AATCCGGCCTTGCAC ATG-3′, RP49F 5′-ATGACCATCCGCCCAGCATCAGG-3′ and RP49R 5′-ATCTCGCCGCAGTAAACG-3′.

Analysis of dSir2 protein levels. Protein was extracted from 30 females at 7 days of age. Western blots were performed using antibodies c-myc 9E10 (Santa Cruz Biotechnology), p2E2 (Developmental Studies Hybridoma Bank) and tubulin (Sigma).

dSir2 deacetylation assays. Sequences encoding *dSir2* (RE27621) and *dSir2–Myc* were cloned into pET SUMO (Invitrogen) and proteins were purified on HisPur cobalt spin columns (Thermo Scientific). For the Fluor de Lys assay, using the SirT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences), results presented are the mean ± s.e.m. of three biological replicates. In each biological replicate, samples were run in triplicate. Final concentrations were: resveratrol and suramin, 0.2 mM; NAD^+ 0.1 mM. Deacetylation of native acetyl-histone-H4 peptide was monitored by HPLC[29]. Deacetylation of histone H4 amino-terminal peptide (SGRGKGGKGLGKGGA(acetyl-K)RHRC) (Biomatik) was carried out using 500 μM NAD^+, 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM dithiothreitol and 0.05% Triton X-100, and monitored by HPLC (Agilent 1100) with an ACE C8-300 150 × 3.0 mm column. The elution profiles were analysed using Chemstation for LC 3D software.

Statistical analysis of *Drosophila* data. Survivorships and the response to dietary restriction were compared using the log-rank test and analyses were performed using JMP, Version 7 (SAS Institute).

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Supplementary information

Figures S1-S12

Tables S1-S7

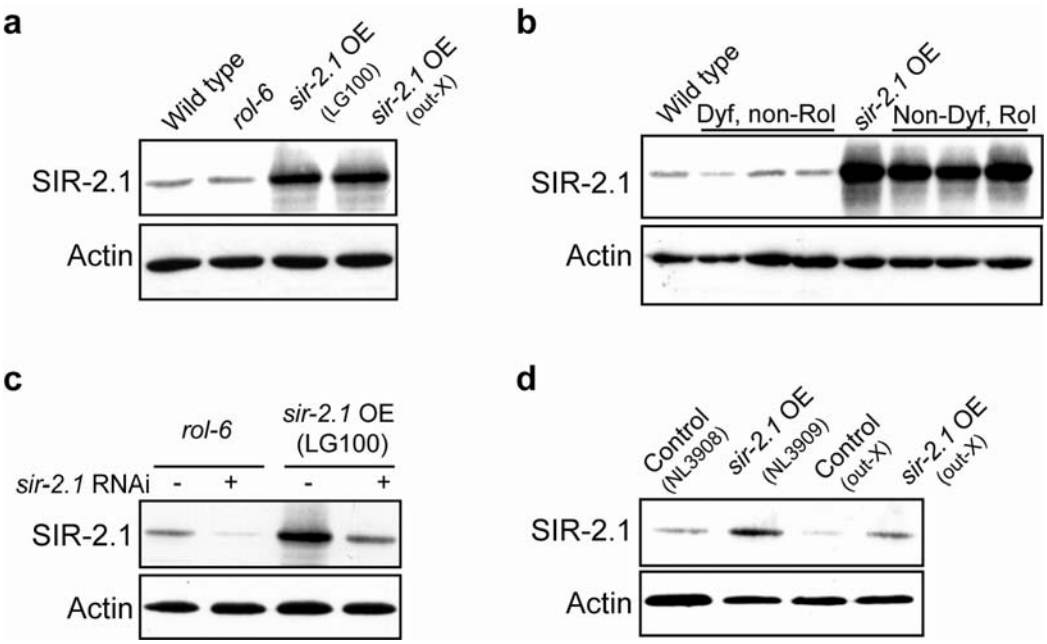


Figure S1 | Western blot analysis of SIR-2 protein levels in transgenic *C. elegans*. **a**, Outcrossing of LG100 does not reduce SIR-2.1 protein levels. Representative example of three trials, for which mean data is shown in Fig. 1b. **b**, Western blot analysis of SIR-2.1 protein levels in Dyf, non-Rol and non-Dyf, Rol lines. Representative example of three trials, for which mean data is shown in Fig. 1d. **c**, *sir-2.1* RNAi reduces SIR-2 protein levels in LG100 to approximately wild-type levels. Representative example of three trials, for which mean data is shown in Fig. 1f. The presence of residual SIR-2.1 protein after RNAi could at least in part reflect *sir-2.1* expression in the subset of neurons that are resistant to RNAi. **d**, Outcrossing of NL3909 does not reduce SIR-2.1 protein levels. Representative example of three trials, for which mean data is shown in Fig. 1h.

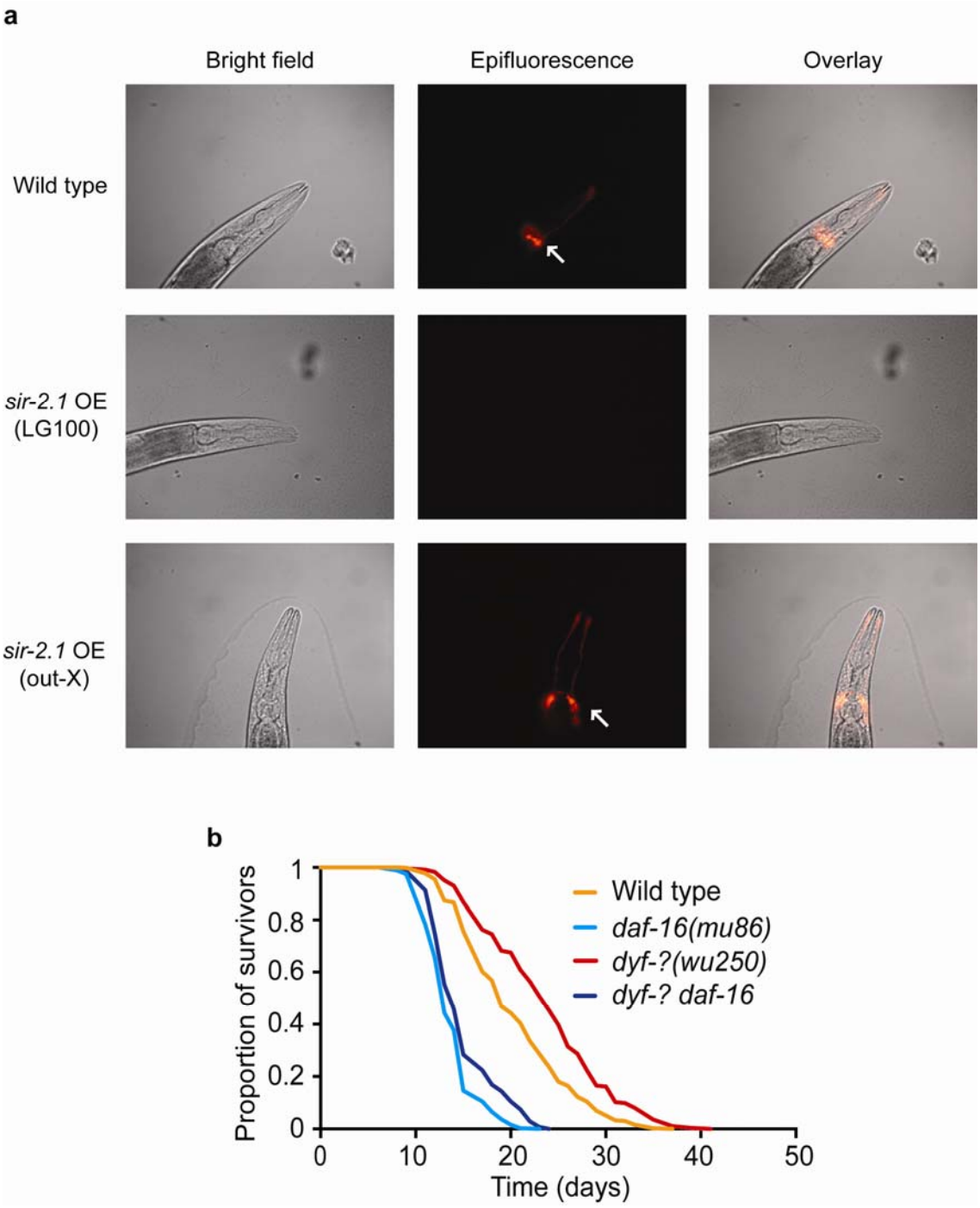


Figure S2 | The LG100 strain contains a dye-filling (*dyf*) mutation. **a**, The outcrossed derivative of LG100 (GA468) shows a normal pattern of amphid neuron staining with the carbocyanin dye DiO (3,3'-dioctadecyloxacarbocyanine perchlorate), while the LG100 strain does not. NL3909 showed a wild-type pattern of amphidial staining with DiO (data not shown). **b**, The increase in lifespan caused by *dyf-?(wu250)* appears to be partially *daf-16* dependent. The following data shows mean lifespan (*N* = sample size [censored values]). CF1038 *daf-16(mu86)* lived 13.71 days (*N* = 460 [86]), GA1114 *dyf-*

?(wu250) daf-16(mu86) 14.96 days (*N* = 213 [276]), GA918 *dyf-?(wu250)* 23.59 (*N* = 366 [154]) and wild type 20.33 (*N* = 515 [54]) (see Figure). *dyf-?(wu250)* increased lifespan by 16.03% in a *daf-16*(+) background (*p* = 0.0001), and by 9.12% in a *daf-16(mu86)* background (*p* = 0.0001). This data combines results of three independent trials, whose results were as follows. Trial 1: CF1038 *daf-16(mu86)* lived 13.74 days (*N* = 136 [14]), GA1114 *dyf-?(wu250) daf-16(mu86)* 13.95 days (*N* = 78 [82]), GA918 *dyf-?(wu250)* 25.01 (*N* = 150 [1]) and wild type 23.70 (*N* = 118 [28]). *dyf-?(wu250)* increased lifespan by 5.5% in a *daf-16*(+) background (*p* = 0.0063), and by 1.53% in a *daf-16(mu86)* background (*p* = 0.44). Trial 2: CF1038 *daf-16(mu86)* lived 14.40 days (*N* = 148 [38]), GA1114 *dyf-?(wu250) daf-16(mu86)* 17.59 days (*N* = 47 [128]), GA918 *dyf-?(wu250)* 26.04 (*N* = 148 [38]) and wild type 21.84 (*N* = 148 [41]). *dyf-?(wu250)* increased lifespan by 19.23% in a *daf-16*(+) background (*p* = 0.0001), and by 22.15% in a *daf-16(mu86)* background (*p* = 0.0001). Trial 3: CF1038 *daf-16(mu86)* lived 13.10 days (*N* = 176 [34]), GA1114 *dyf-?(wu250) daf-16(mu86)* 14.22 days (*N* = 88 [66]), GA918 *dyf-?(wu250)* 19.05 (*N* = 138 [20]) and wild type 16.75 (*N* = 217 [12]). *dyf-?(wu250)* increased lifespan by 13.7% in a *daf-16*(+) background (*p* = 0.0001), and by 8.55% in a *daf-16(mu86)* background (*p* = 0.0005). Thus, in two out of three trials, *daf-16(mu86)* reduced the magnitude of the increase in mean lifespan caused by *dyf-?(wu250)*. Trials performed at 20˚C.

Previously, we mapped *dyf-?(wu250)* to the right of the *unc-13* locus on the right arm of LG I (data not shown). Construction of a *daf-16(mu86) dyf-?(wu250)* strain provided further 2 point mapping data, as follows. *dyf-?(wu250)* +/+ *daf-16(mu86)* animals were selfed, and 83 Dyf F1s plated individually and allowed to lay eggs. The F1 mothers were then PCR genotyped, and 4/83 proved to be *mu86*/+. This places *dyf-?* ~2.40 map units away from *daf-16*. *che-3* is 2.63 map units to the left of *daf-16*. However, worms heterozygous for both *dyf-?(wu250)* and *che-3(p801)* were not Dyf, suggesting (though not proving) that *wu250* is not an allele of *che-3*.

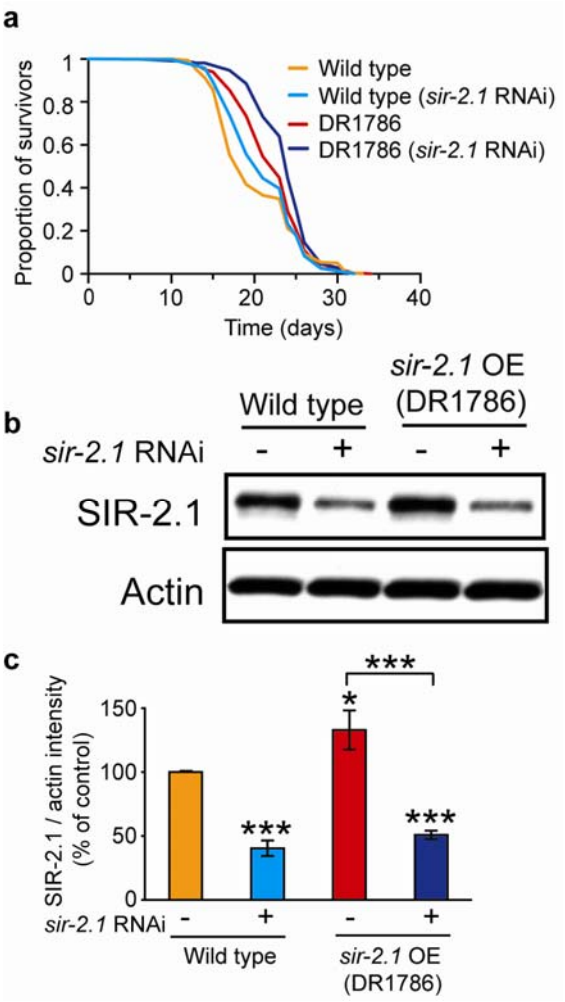


Figure S3 | Longevity of DR1786 is not *sir-2.1* dependent. DR1786 is a long-lived strain containing the *mDp4* duplication, which includes the *sir-2.1* locus. **a**, DR1786 is longer lived than wild type, and this longevity is not suppressed by *sir-2.1* RNAi. See Table S7 for full statistical analysis. **b, c**, *sir-2.1* RNAi reduces levels of SIR-2.1 protein (Western blot analysis). Note that in the absence of RNAi, SIR-2.1 protein levels are significantly elevated in DR1786 relative to wild type. * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$; Student's *t* test (two tailed).

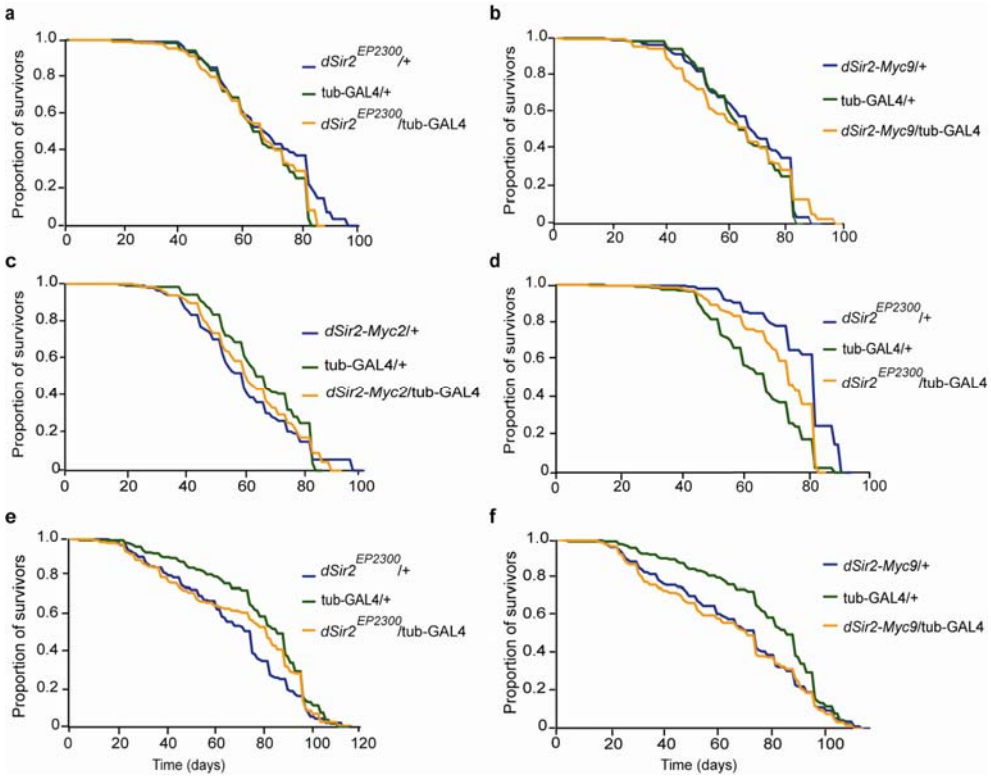


Figure S4 | Lifespan is not increased in flies over-expressing *dSir2* on any media type. All lines were outcrossed into w^{Dah} (+/+). **(a-c)** Ubiquitous over-expression of *dSir2* via EP2300, *dSir2-Myc9* or *dSir2-Myc2* does not increase lifespan on SYA media lacking propionic acid over that of the controls. **a**, Median lifespans: $dSir2^{EP2300}$/tubulin-GAL4 = 65 days, $dSir2^{EP2300}$/+ = 66 days, tubulin-GAL4/+ = 65 days. $dSir2^{EP2300}$/tubulin-GAL4 *vs*. $dSir2^{EP2300}$/+, P = 0.14. $dSir2^{EP2300}$/tubulin-GAL4 *vs.* tubulin-GAL4/+, P = 0.69. **b**, Median lifespans: *dSir2-Myc9*/tubulin-GAL4 = 65 days, *dSir2-Myc9*/+ = 67 days tubulin-GAL4/+= 65 days. *dSir2-Myc9*/tubulin-GAL4 *vs*. *dSir2-Myc9*/+, P = 0.27. *dSir2-Myc9*/tubulin-GAL4 *vs*. tubulin-GAL4/+, P = 0.95. **c**, Median lifespans: *dSir2-Myc2*/tubulin-GAL4 = 59 days, *dSir2-Myc2*/+ = 58 days, tubulin-GAL4/+ = 65 days. *dSir2-Myc2*/tubulin-GAL4 *vs*. *dSir2-Myc2*/+, P = 0.23. *dSir2-Myc2*/tubulin-GAL4 *vs*. tubulin-GAL4/+, P = 0.077. **d**, Over-expression of *dSir2* does not increase lifespan on SYA media containing propionic acid. Median lifespans: $dSir2^{EP2300}$/tubulin-GAL4 = 72 days, $dSir2^{EP2300}$/+ = 80 days, tubulin-GAL4/+ = 65 days. $dSir2^{EP2300}$/tubulin-GAL4 *vs*. $dSir2^{EP2300}$/+, P <0.0001. $dSir2^{EP2300}$/tubulin-GAL4 *vs*. tubulin-GAL4/+, P < 0.0001. **(e, f)** Over-expression of *dSir2* does not increase lifespan on ASG media containing maize. **e**, Median lifespans: $dSir2^{EP2300}$/tubulin-GAL4 = 79 days, $dSir2^{EP2300}$/+= 72 days, tubulin-GAL4/+ = 86 days. $dSir2^{EP2300}$/tubulin-GAL4 *vs*. $dSir2^{EP2300}$/+, P = 0.032). $dSir2^{EP2300}$/tubulin-GAL4 *vs*. tubulin-GAL4/+, P = 0.037. **f**, Median lifespans: *dSir2-Myc9*/tubulin-GAL4 = 69 days, *dSir2-Myc9*/+ = 72 days, tubulin-GAL4/+ = 86 days. *dSir2-Myc9*/tubulin-GAL4 *vs*. *dSir2-Myc9*/+, P = 0.57. *dSir2-Myc9*/tubulin-GAL4 *vs*. tubulin-GAL4/+, P <0.0001.

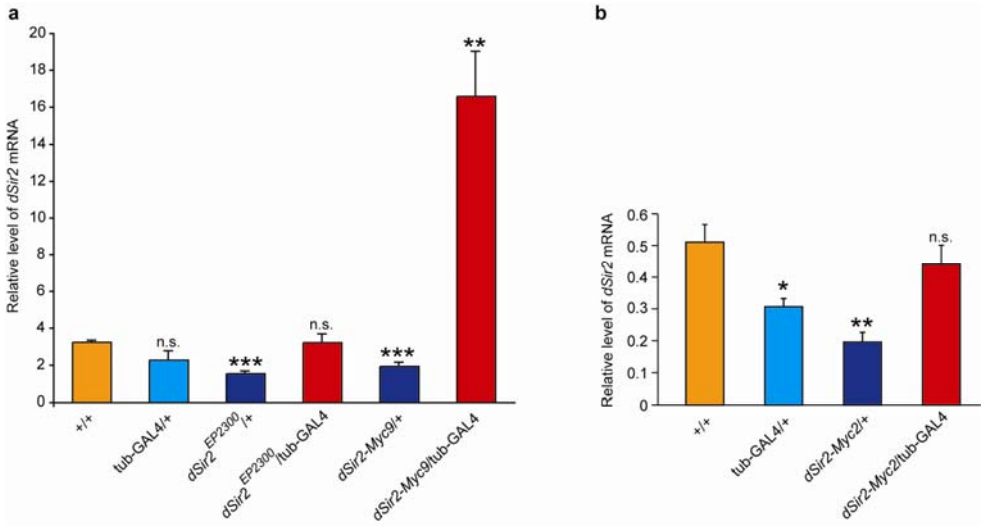


Figure S5 | *dSir2* mRNA levels in transgenic *Drosophila* lines, driven by tubulin-GAL4. **a**, Expression from $dSir2^{EP2300}$ and *dSir2-Myc9*. The insert $dSir2^{EP2300}$ reduces *dSir2* mRNA levels to below that of wild type, w^{Dah} (+/+) (P = 2.5 x10^{-5}), suggesting that this fly line is hypomorphic for *dSir2*. Over-expression of $dSir2^{EP2300}$ significantly increases *dSir2* mRNA levels (P = 0.0105) but only to the same level as the wild type (P = 0.957). The presence of *dSir2-Myc9* also decreases *dSir2* (P = 0.001) but driving over-expression of this insert significantly increases levels of *dSir2* compared to levels in wild type (P = 0.001). **b**, Expression from *dSir2-Myc2*. The insert *dSir2-Myc2* reduces *dSir2* mRNA levels to below that of wild type, w^{Dah} (+/+), (P = 0.0017) suggesting that this fly line is also hypomorphic for *dSir2*. Over-expression of *dSir2-Myc2* significantly increases *dSir2* mRNA levels but only to the same level as the wild type (P = 0.63). Normalised to Actin5C. These results were repeated and confirmed by normalisation to a second housekeeping gene, RP49 (data not shown). n.s., not significant (comparison to wild type). Error bars, S.E.M.. n.s., not significant. * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001; Student's *t* test (two tailed).

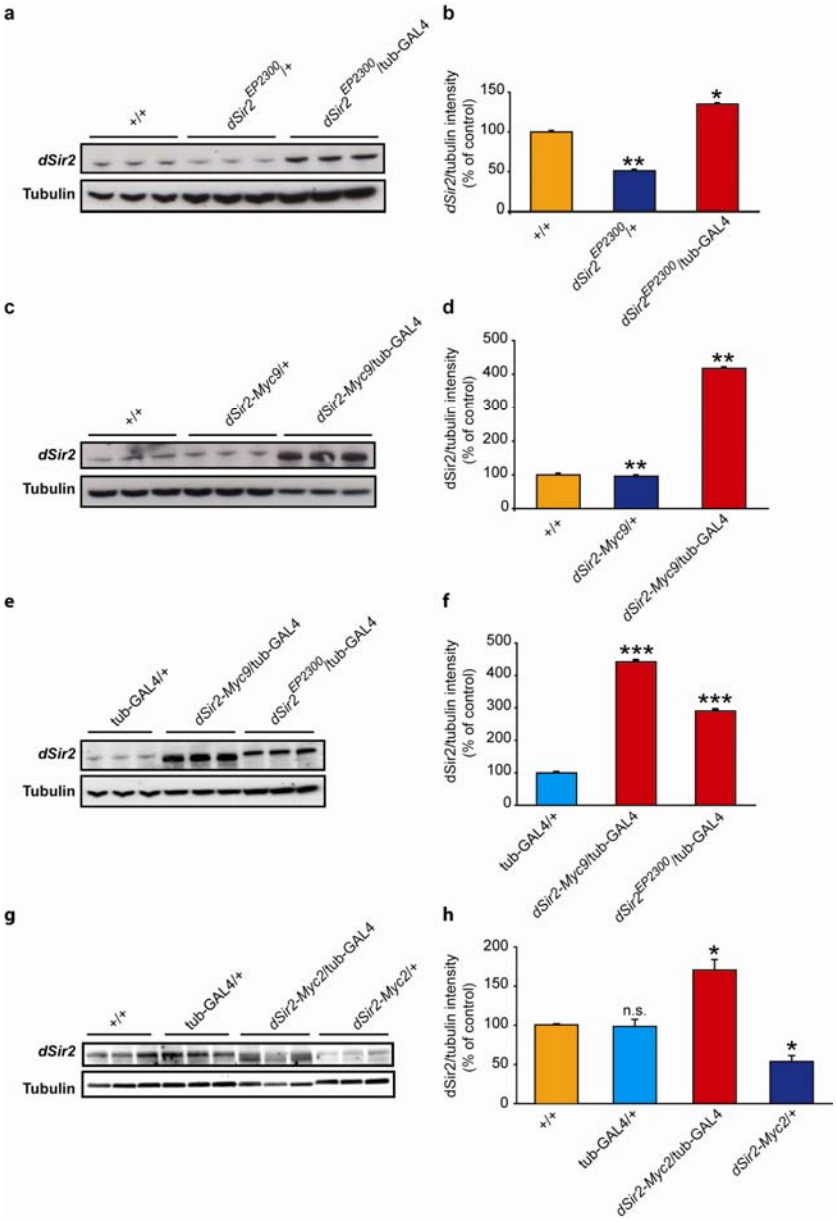


Figure S6 | Over-expression of *dSir2* in fly lines driven by tubulin-GAL4, detected with a polyclonal antibody to *dSir2*. **a**, **b**, *dSir2* protein levels are increased by 35% relative to wild-type in $dSir2^{EP2300}$/tubulin-GAL4 (P = 0.0199) (densitometric analysis of Western blots). The control line $dSir2^{EP2300}$/+ has only 51% of wild-type *dSir2* levels (P = 0.0038) and 38% of $dSir2^{EP2300}$/tubulin-GAL4 levels (P = 0.0002), implying that $dSir2^{EP2300}$ is a hypomorphic allele. **c, d**, *dSir2* levels are increased by 318% in *dSir2-Myc9*/tubulin-GAL4 flies relative to wild-type (P = 0.0043) and 336% relative to the control line *dSir2-Myc9*/+ (P = 0.004). **e, f**, Levels of *dSir2* are increased by 190% in $dSir2^{EP2300}$/tubulin-GAL4 (P = 0.0001) and 342% in *dSir2-Myc9*/tubulin-GAL4 ($P = 2.4\text{x}10^{-7}$) relative to tubulin-GAL4 /+ which has only 65% of wild-type *dSir2* levels (P = 0.0233). **g, h**, *dSir2* levels are increased by 71% in *dSir2-Myc2*/tubulin-GAL4 flies relative to wild-type (P = 0.0102), by 73% in *dSir2-Myc2*/tubulin-GAL4 flies relative to tubulin-GAL4/+ (P = 0.0113), and 217% relative to the control line *dSir2-Myc9*/+ (P = 0.0016). Error bars, S.E.M.. *0.01 < P < 0.05; ** 0.001 < P < 0.01; ***P < 0.001; Student's t test (two tailed).

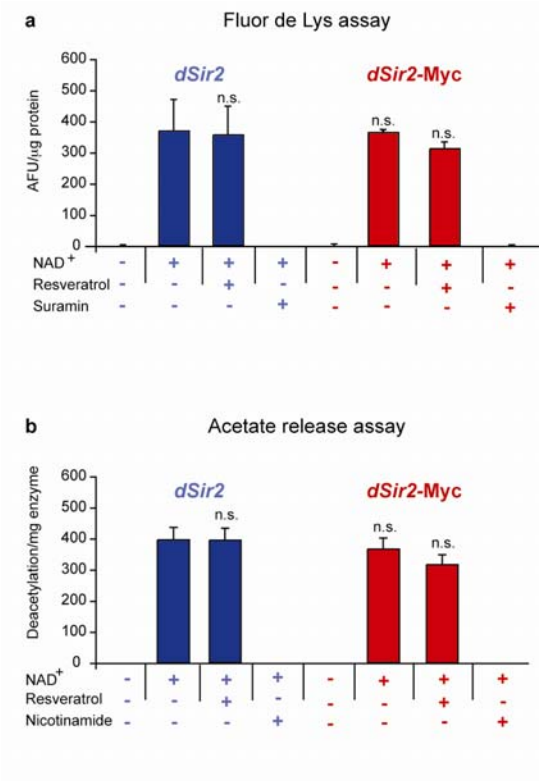


Figure S7 | The enzymatic activity of *dSir2* is not affected by the C-terminal Myc tag, or by resveratrol. **a**, Purified recombinant protein was run in the BioMol *Flour de Lys* assay. Results are the combined means of three biological replicates, each run in triplicate, ± S.E.M. Resveratrol does not activate *dSir2* under these conditions. Addition of an inhibitor or the lack of the cofactor NAD^+ abolishes activity (data from UCL). AFU, arbitrary fluorescence units. **b**, Deacetylation of native acetyl-histone H4 peptide monitored by HPLC. All reactions were run in triplicate. Representative of three trials with similar results (data from the Fred Hutchinson Cancer Research Center). At both research sites, resveratrol increased SirT1 HDAC activity in the *Flour de Lys* assay (data not shown) as expected[1], confirming the bioactivity of the resveratrol used. Error bars, S.E.M.. n.s., not significant; Student's *t* test (two tailed).

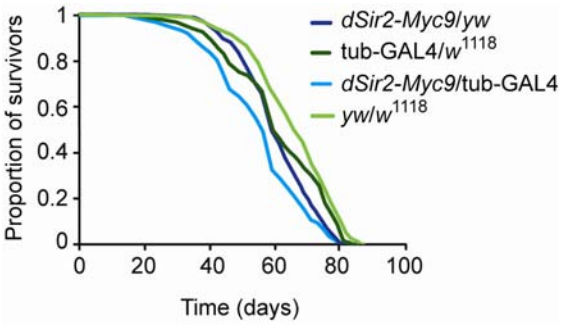


Figure S8 | Over-expression of *dSir2*-Myc does not increase *Drosophila* lifespan. Lifespan in flies over-expressing *dSir2-Myc9* is not increased relative to any of the genetic controls on 15% SYA media. Consistent with findings in Fig. 1b. *dSir2-Myc9* were outcrossed into w^{1118} 8 times, tubulin-GAL4 were outcrossed into *yw* 20 times. Median lifespans: *dSir2-Myc9*/tub-GAL4 = 55 days, *dSir2-Myc9*/*yw* = 61 days, tubulin-GAL4/w^{1118} = 61 days, *yw*/w^{1118} = 67 days. *dSir2-Myc9*/tub-GAL4 *vs. yw*/w^{1118}, $P = 1.4 \times 10^{-5}$; *dSir2-Myc9*/tub-GAL4 *vs. dSir2-Myc9*/*yw*, $P = <1 \times 10^{-10}$; *dSir2-Myc9*/tub-GAL4 *vs.* Tubulin-GAL4/w^{1118}, $P = 9.4 \times 10^{-9}$. *yw* and w^{1118} are alternative wild type strains. We are grateful to Danielle Skorupa and Scott Pletcher (University of Michigan Medical School) for providing this data.

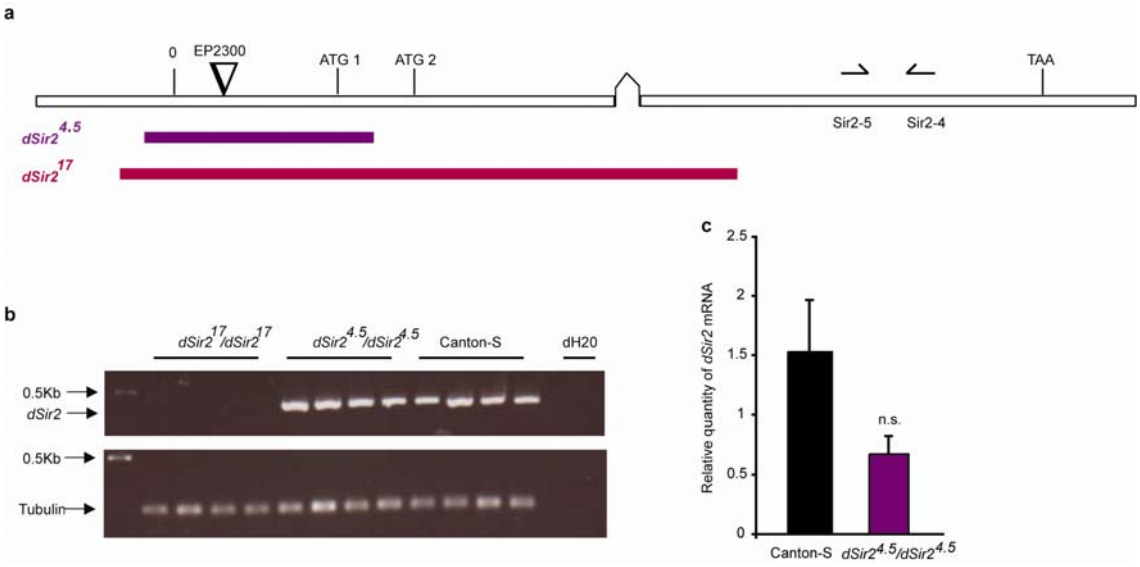


Figure S9 | Effects of *dSir2* deletion alleles on *dSir2* mRNA levels. **a**, Genomic organization of *dSir2*. The extent of both deletions are shown, as previously defined for $Sir2^{4.5}$ (Ref. 2) and $dSir2^{17}$ (Ref. 3). 0, putative transcriptional start site, as previously defined by primer extension analysis[2]. Also shown are the locations of the oligonucleotide primers (Sir2-4 and Sir2-5) used here, and previously[4], for RT-PCR amplification of the *sir-2.1* mRNA. **b**, RT-PCR and **c**, QRT-PCR measurements of *dSir2* mRNA (each from 4 biological samples). Error bars, S.E.M.. These results are consistent with $dSir2^{17}$ being a null allele. By contrast, *dSir2* mRNA levels in $dSir2^{4.5}$ are indistinguishable from wild type (P = 0.110) . The ATG that is still present in $dSir2^{4.5}$ is the start codon for the *dSir2-Myc* protein. n.s., not significant; Student's *t* test (two tailed).

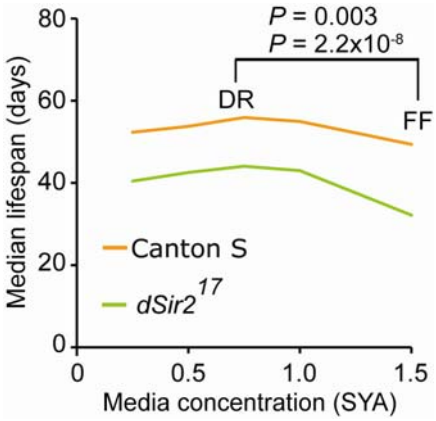


Figure S10 | Effect of dietary restriction on *Drosophila* lifespan is not *dSir2* dependent. Replicate experiment performed at the University of Michigan. Flies were assayed over five concentrations of SYA media and data are presented as the median lifespan on each food concentration. All lines were outcrossed into Canton S (+/+). *P* values confirm that all flies respond normally to DR when median lifespans are compared for DR *vs.* fully-fed (FF) conditions. Results are consistent with those in Fig. 2C insofar as absence of dSir2 has no effect on the response to DR which, if anything, is stronger in $dSir2^{17}$(P=2.2x10^{-8}) than in Canton S (P=0.003). However, in this trial, the effect of DR on lifespan in both strains was relatively modest, and the $dSir2^{17}$ mutant was slightly shorter lived at all food concentrations. $dSir2^{4.5}$ flies responded similarly to DR (not shown). We are grateful to Scott Pletcher (University of Michigan Medical School) for providing this data.

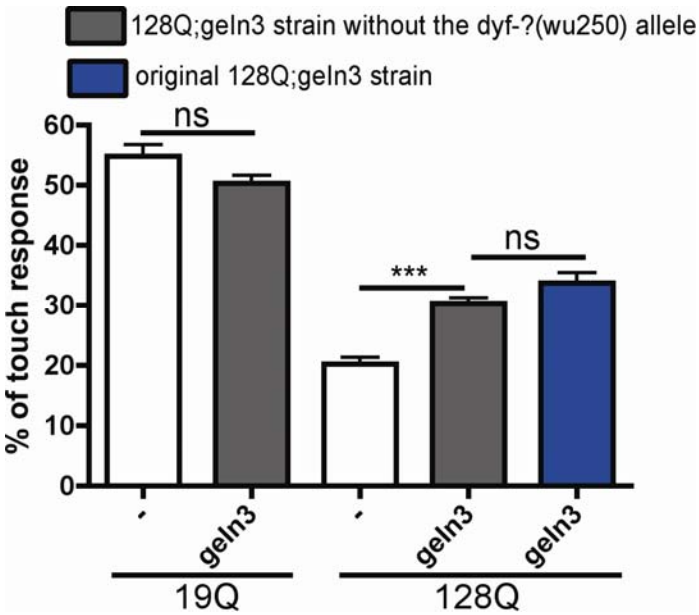
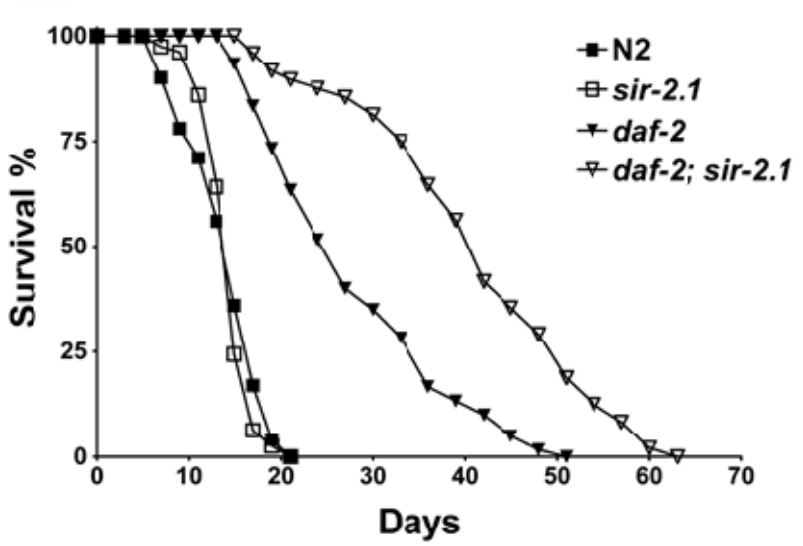


Figure S11 | Increased *sir-2.1* dosage protects neurons from expanded polyQs. Protection previously reported[5] is not attributable to the *dyf-?(wu250)* mutation. Analysis of touch response in the tail of transgenic *C. elegans* expressing wild-type (19Q) or expanded (128Q) N-terminal huntingtin in touch receptor neurons (controls), with or without *geIn3* (increased *sir-2.1* dosage). Neuronal dysfunction caused by 128Q was ameliorated by *geIn3* in worms in which *dyf-?(wu250)* was confirmed not to be present. The level of neuroprotection conferred by *geIn3* dissociated from *dyf-?(wu250)* was similar to that previously reported[5] in 128Q animals crossed to the original LG100 strain. Results are mean ± SEM. ***P < 0.0001. n.s., not significant. (Data from Inserm, Université Paris Descartes).



Genotype	Lifespan (days) mean	max	Percent of control	n	*p*
Experiment 1					
N2	14.0	21	-	100	-
sir-2.1(ok434)	14.5	21	104%	78	0.58
daf-2(e1370)	28.0	51	200%	60	<0.0001
daf-2; sir-2.1	41.4	63	296%	48	<0.0001
Experiment 2					
N2	13.9	21	-	111	-
sir-2.1(ok434)	14.0	19	101%	69	0.94
daf-2(e1370)	27.7	51	199%	114	<0.0001
daf-2; sir-2.1	37.8	60	272%	93	<0.0001

Figure S12 | Deletion of *sir-2.1* increases *C. elegans* lifespan in a *daf-2(e1370)* background. *daf-2(e1370)* is a class 2 (pleiotropic) *daf-2* allele which leads to hypersensitivity to genetic effects on lifespan[6]. This data confirms findings in an earlier report[7]. Animals were grown at 15˚C until the late L4 stage, and then transferred to 25˚C for lifespan measurements. We are grateful to Di Chen and Pankaj Kapahi (Buck Institute for Age Research) for providing this data.

References for supplemental figure legends

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[4] Rogina, B. & Helfand, S. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci USA* **101**, 15998-16003 (2004).

[5] Parker, J. A. *et al.* Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. *Nat Genet* **37**, 349-350 (2005).

[6] Patel, D. S. *et al.* Clustering of genetically defined allele classes in the *Caenorhabditis elegans* DAF-2 insulin/IGF-1 receptor. *Genetics* **178**, 931-946 (2008).

[7] Berdichevsky, A., Viswanathan, M., Horvitz, H. R. & Guarente, L. *C. elegans* SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. *Cell* **125**, 1165-1177 (2006).

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Table S1. Effect of outcrossing on lifespan in high-copy number *sir-2.1* over-expressing *C. elegans* strain

Strain, genotype	Number of deaths/ censored[1]	Mean life span (days)	% change *vs.* N2	*p vs.* wild type (log rank)	% change *vs.* GA707	*p vs.* GA707	% change *vs.* LG100	*p vs.* LG100 (log rank)
N2 (wild type)	**[C] 598/228**	**20.41**						
	[1] 84/43	18.89						
	[2] 79/48	19.91						
	[3] 62/63	22.60						
	[4] 86/34	19.03						
	[5] 164/9	21.05						
	[6] 123/31	20.11						
	[C_1] 224/110	**21.13**						
	[7] 104/82	20.29						
	[8] 120/28	22.10						
GA707 *wuEx166 [rol-6(su1006)]*	**[C] 440/226**	**20.53**	**+0.58**	**0.66**				
	[1] 81/49	21.04	+11.38	0.01				
	[2] 96/29	21.79	+9.44	<.0001				
	[3] 75/50	23.64	+4.60	0.13				
	[4] 63/53	17.06	-10.35	0.0007				
	[5] 44/19	18.45	-12.35	0.003				
	[6] 81/79	19.19	-4.57	0.10				
LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*	**[C] 678/115**	**25.57**	**+25.28**	**<.0001**	**+24.54**	**<.0001**		
	[1] 91/17	26.57	+41.70	<.0001	+26.28	<.0001		
	[2] 119/7	31.75	+59.46	<.0001	+45.70	<.0001		
	[3] 116/6	26.25	+16.15	0.0001	+8.60	0.002		
	[4] 106/29	21.93	+15.23	<.0001	+28.54	<.0001		
	[5] 146/7	24.93	+18.43	<.0001	+35.12	<.0001		
	[6] 100/49	21.13	+5.07	0.02	+10.10	0.004		
GA468 *geIn3 [sir-2.1 rol-6(su1006)]* (Outcrossed x5)	**[C] 523/324**	**20.48**	**+0.34**	**0.90**	**-0.24**	**0.82**	**-19.90**	**<.0001**
	[1] 77/48	18.75	-0.74	0.91	-10.88	0.0007	-29.43	<.0001
	[2] 71/64	21.67	+8.83	0.0007	-0.55	0.67	-31.74	<.0001
	[3] 66/59	21.13	-6.50	0.27	-12.57	0.002	-19.50	<.0001
	[4] 68/75	17.79	-6.51	0.01	+4.27	0.14	-18.87	<.0001
	[5] 167/9	21.50	+2.13	0.83	+16.53	0.0006	-13.75	<.0001
	[6] 74/69	19.65	-2.28	0.82	+2.39	0.28	-7.00	0.03
	[C_1] 276/35	**20.56**	**-2.69**	**0.17**				
	[7] 137/21	20.02	-1.33	0.49				
	[8] 139/14	21.10	-4.52	0.27				

[1][C], combined data with FUdR, [C_1], combined data without FUdR, [n], trial number. Trials performed at 20˚C, using 40μM FUdR [trials 1-6] to prevent progeny production, or without FUdR [trials 7,8]. This, and all other data was generated at UCL, unless otherwise stated.

Table S2. Effect of outcrossing on lifespan on high-copy number *sir-2.1* over-expressing *C. elegans* strain (data from University of Washington)

Strain, genotype	**Number of deaths/ censored**	**Mean life span (days)**	**% change *vs.* N2**	***p vs.* wild type (log rank)**	**% change *vs.* CL3099**	***p vs.* CL2099**	**% change *vs.* LG100**	***p vs.* LG100 (log rank)**
N2 (wild type)	105/0	25.15						
CL2099 *dvIs22 [rol-6 (su1006)]*	110/0	24.71	-1.74	0.62				
LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*	112/0	30.26	+20.31	<.0001	+22.46	<.0001		
MK287 *geIn3 [sir-2.1 rol-6(su1006)]* (OC x6)	118/0	23.55	-6.36	0.01	-4.69	0.14	-22.17	<.0001
MK288 *geIn3 [sir-2.1 rol-6(su1006)]* (OC x6)	106/0	22.53	-10.41	<.0001	-8.82	0.0007	-25.54	<.0001
MK289 *geIn3 [sir-2.1 rol-6(su1006)]* (OC x6)	121/0	22.47	-10.65	<.0001	-9.06	0.0002	-25.74	<.0001

Trials performed at 20˚C, using 50μM FUDR to prevent progeny production, and UV-irradiated *E. coli* to block its pathogenic effects; methodology as previously described[8].

Table S3. Longevity of *C. elegans* LG100 strain segregates with *dyf-?(wu250) I* mutation

Strains	Number of deaths/ censored	Mean life span	% *vs.* wildtype	*p vs.* wildtype	% *vs.* GA707	*p vs.* GA707	% *vs.* LG100	*p vs.* LG100
Wildtype	**[C] 250/43**	**20.37**						
	[1] 86/34	19.03						
	[2] 164/9	21.05						
GA707	**[C] 107/72**	**17.65**	**-13.35**	**<.0001**				
	[1] 63/53	17.06	-10.35	0.0007				
	[2] 44/19	18.45	-12.35	0.003				
LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*	**[C] 252/36**	**23.66**	**+16.15**	**<.0001**	**+34.05**	**<.0001**		
	[1] 106/29	21.93	+15.23	<.0001	+28.54	<.0001		
	[2] 146/7	24.93	+18.43	<.0001	+35.12	<.0001		
Rol, non-Dyf lines	**[C] 560/174**	**19.42**	**-4.66**	**0.0006**	**+10.02**	**<.0001**	**-17.92**	**<.0001**
GA919 *geIn3 [sir-2.1 rol-6(su1006)]*	**[C] 207/87**	**18.33**	**-10.01**	**<.0001**	**+3.85**	**0.09**	**-22.52**	**<.0001**
	[1] 54/75	16.10	-15.39	<.0001	-5.62	0.11	-26.58	<.0001
	[2] 153/12	19.01	-9.69	<.0001	+3.03	0.76	-23.74	<.0001
GA920 *geIn3 [sir-2.1 rol-6(su1006)]*	[2] 125/21	21.58	+2.51	0.81	+16.96	0.001	-13.43	<.0001
GA921 *geIn3 [sir-2.1 rol-6(su1006)]*	**[C] 228/66**	**19.17**	**-5.89**	**<.0001**	**+8.61**	**0.002**	**-18.97**	**<.0001**
	[1] 95/55	17.36	-8.77	0.0004	+1.75	0.75	-20.83	<.0001
	[2] 133/11	20.35	-3.32	0.003	+10.29	0.06	-18.37	<.0001
Dyf, non-Rol lines	**[C] 647/256**	**23.45**	**+15.12**	**<.0001**	**+32.86**	**<.0001**	**-0.88**	**0.79**
GA916 *dyf-?(wu250)*	**[C] 204/91**	**23.17**	**+13.74**	**<.0001**	**+31.27**	**<.0001**	**-2.07**	**0.81**
	[1] 55/80	20.45	+7.46	0.05	+19.87	<.0001	-6.74	0.07
	[2] 149/11	24.07	+14.34	0.0001	+30.46	<.0001	-3.44	0.91
GA917 *dyf-?(wu250)*	**[C] 238/57**	**22.80**	**+11.92**	**0.0001**	**+29.17**	**<.0001**	**-3.63**	**0.36**
	[1] 107/46	20.99	+10.29	0.005	+23.03	<.0001	-4.28	0.12
	[2] 131/11	24.14	+14.67	0.0001	+30.84	<.0001	-3.16	0.93
GA918 *dyf-?(wu250)*	**[C] 205/108**	**24.39**	**+19.73**	**<.0001**	**+38.18**	**<.0001**	**+3.08**	**0.19**
	[1] 77/86	22.97	+20.70	<.0001	+34.64	<.0001	+4.74	0.10
	[2] 128/22	25.29	+20.14	<.0001	+37.07	<.0001	+1.44	0.84

Trials performed at 20˚C, using 10μM FUdR to prevent progeny production. [1][C], combined data. Strains GA916-GA921 are derived from true-breeding F2 segregants of LG100 crossed with N2; their genotypes were deduced from the phenotypes described. A formal possibility is that *dyf-?(wu250)* sensitizes worms to the longevity-inducing effects of *geIn3*. However, the fact the three Dyf, non-Rol derivatives, GA916, GA917 and GA918 are not shorter lived than LG100 argues against this.

Table S4. Effect of *sir-2.1* RNAi on lifespan in high-copy number *sir-2.1* over-expressing *C. elegans* strains

Strain, genotype	RNAi treatment	Number of deaths/ censored[1]	Mean life span (days)	% change *vs.* N2 on L4440	*p vs.* N2 on L4440 (log rank)	% change *vs.* GA707 on L4440	*p vs.* GA707 on L4440 (log rank)	% change *vs.* same strain on L4440	*p vs.* same strain on L4440 (log rank)
N2 (wild type)	L4440	**[C] 210/11**	**18.66**						
		[1] 118/9	17.87						
		[2] 92/2	19.67						
N2 (wild type)	*sir-2.1*	**[C] 166/74**	**18.42**	**-1.28**	**0.44**	**-0.27**	**0.52**		
		[1] 117/8	18.17	+1.68	0.45	-1.35	0.22		
		[2] 49/66	19.08	– 2.99	0.36	+3.07	0.33		
GA707 *wuEx166 [rol-6 (su1006)]*	L4440	**[C] 194/47**	**18.47**	**-0.53**	**0.50**				
		[1] 97/28	18.42	+3.07	0.07				
		[2] 97/19	18.51	-5.89	0.02				
GA707 *wuEx166 [rol-6 (su1006)]*	*sir-2.1*	**[C] 177/71**	**19.38**	**+3.35**	**0.02**	**+4.92**	**0.006**		
		[1] 107/16	19.13	+7.05	0.0004	+5.28	0.04		
		[2] 68/55	19.41	-1.32	0.77	+4.86	0.08		
LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*	L4440	**[C] 222/25**	**20.93**	**+12.16**	**<.0001**	**+13.31**	**<.0001**		
		[1] 117/9	19.78	+8.50	<.0001	+6.88	0.01		
		[2] 105/16	22.25	+13.11	<.0001	+20.20	<.0001		
LG100 *geIn3 [sir-2.1 rol-6(su1006)] dyf-?(wu250)*	*sir-2.1*	**[C] 207/44**	**21.91**	**+17.41**	**<.0001**	**+18.62**	**<.0001**	**+4.68**	**0.06**
		[2] 108/17	21.06	+16.51	<.0001	+14.58	<.0001	+6.47	0.05
		[3] 99/27	22.68	+ 15.30	<.0001	+22.52	<.0001	+1.93	0.29

Trials performed at 20˚C (no FUDR). [1][C], combined data. L4440 is RNAi plasmid vector (negative control).

Table S5. Effect of outcrossing on lifespan in low-copy number *sir-2.1* over-expressing line

Strain, genotype	Number of deaths/ censored[1]	Mean life span (days)	% change *vs.* N2	*p vs.* N2 (log rank)	% change *vs.* NL3909	*p vs.* NL3909 (log rank)	% change *vs.* GA905	*p vs.* GA905 (log rank)	% change *vs.* non-out-crossed strain	*p vs.* non-out-crossed strain (log rank)
N2 (wild type)	**[C] 507/368**	**19.46**								
	[1] 58/68	17.12								
	[2] 90/28	17.39								
	[3] 106/19	19.44								
	[4] 84/41	18.75								
	[5] 79/48	19.91								
	[6] 90/162	22.60								
	[C_1] 224/110	**21.13**								
	[7] 104/82	20.29								
	[8] 120/28	22.10								
NL3909 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)*	**[C] 619/130**	**22.62**	**+16.23**	**<.0001**						
	[1] 114/13	21.90	+27.92	<.0001						
	[2] 117/11	21.45	+23.34	<.0001						
	[3] 110/9	21.80	+ 12.13	0.0006						
	[4] 119/5	23.30	+24.26	<.0001						
	[5] 114/12	24.04	+20.74	<.0001						
	[6] 45/80	24.17	+6.94	0.09						
GA905 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)* (outcrossed x6)	**[C] 291/88**	**17.02**	**-12.53**	**<.0001**					**-24.75**	**<.0001**
	[1] 91/39	16.12	-5.84	0.01					-26.39	<.0001
	[2] 97/23	17.62	+1.32	0.53					-17.85	<.0001
	[3] 103/26	17.30	-11	0.003					-20.64	<.0001
	[C_1] 246/35	**19.99**	**-5.39**	**0.03**						
	[7] 142/24	19.07	-6.01	0.08						
	[8] 104/11	21.24	-3.89	0.41						
NL3908 *pkIs1641 [unc-119] unc-119(ed3)*	**[C] 530/107**	**19.15**	**-1.59**	**0.30**	**-15.34**	**<.0001**				
	[1] 113/19	19.92	+16.35	<.0001	-9.04	0.0004				
	[2] 118/10	17.27	-0.69	0.89	-19.48	<.0001				
	[3] 99/19	17.06	-12.24	0.003	-21.74	<.0001				
	[4] 104/21	20.05	+6.93	0.09	-13.94	<.0001				
	[5] 96/38	21.37	+7.33	0.0007	-11.10	<.0001				
	[6] 28/99	22.44	-0.70	0.31	-7.15	0.02				
GA906 *pkIs1641 [unc-119] unc-119(ed3)* (outcrossed x6)	**[C] 275/105**	**16.55**	**-14.95**	**<.0001**			**-2.76**	**0.40**	**-13.57**	**<.0001**
	[1] 77/47	15.90	-7.12	0.004			-1.36	0.58	-20.18	<.0001
	[2] 97/23	16.82	-3.27	0.53			-4.54	0.32	-2.60	0.63
	[3] 112/13	16.89	-13.11	0.001			-2.36	0.60	-0.99	0.76
	[C_1] 241/57	**20.43**	**-3.31**	**0.29**			**+2.20**	**0.24**		
	[7] 131/31	19.03	-6.20	0.14			-0.20	0.62		
	[8] 110/26	22.23	+0.58	0.82			-4.45	0.29		

[1][C], combined data with FUdR, [C_1], combined data without FUdR, [n], trial number. Trials performed at 20˚C, using 40μM FUdR [trials 1-6] to prevent progeny production, or without FUdR [trials 7,8].

Table S6. Effect of outcrossing on lifespan in low-copy number *sir-2.1* over-expressing line (data from Semmelweis University)

Strain, genotype	**Number of deaths/ censored**	**Mean life span (days)**	**% change *vs.* N2**	***p vs.* N2 (log rank)**	**% change *vs.* NL3908**	***p vs.* NL3909 (log rank)**	**% change *vs. pkIs1641* outcrossed**	***p vs. pkIs1641* outcrossed**	**% change *vs.* outcrossed strain**	***p vs.* outcrossed strain (log rank)**
N2 (wild type)	55/18	22.13								
NL3909 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)*	60/10	27.53	+24.39	<.0005	+16.16	0.002			+16.30	0.01
SCS003 *pkIs1642 [sir-2.1 unc-119] unc-119(ed3)* (OCx6)	43/8	23.67	+6.94	0.10			+2.51	0.24		
NL3908 *pkIs1641 [unc-119] unc-119(ed3)*	61/9	23.70	+7.11	0.07					+2.64	0.32
SCS004 *pkIs1641 [unc-119] unc-119(ed3)* (OCx6)	56/3	23.09	+4.35	0.26						

Trials performed at 20˚C, using 50μM FUDR to prevent progeny production. We also attempted to perform *sir-2.1* RNAi on NL3909, but found that on HT115/L4440 NL3909 was not longer lived than NL3908 (effect seen both at Semmelweis University and UCL) (data not shown).

Table S7: Effect of *sir-2.1* RNAi on life span in the *C. elegans* duplication strain DR1786

Strain	RNAi	Deaths/ censored	Mean life span (days)	% *vs.* wildtype ctrl	*p vs.* wildtype ctrl	% *vs.* DR1786 ctrl	*p vs.* DR1786 ctrl
Wild type	Control	**[C] 295/12**	**20.33**				
		[1] 175/7	21.50				
		[2] 120/5	18.61				
Wild type	*sir-2.1*	**[C] 262/58**	**21.23**	**+4.42**	**0.16**		
		[1] 117/49	23.12	+7.53	0.02		
		[2] 145/9	19.29	+3.65	0.27		
DR1786	Control	**[C] 330/55**	**22.30**	**+9.69**	**0.0005**		
		[1] 170/41	22.15	+3.02	0.92		
		[2] 160/14	22.46	+20.68	<.0001		
DR1786	*sir-2.1*	**[C] 288/70**	**23.80**	**+17.06**	**<.0001**	**+6.72**	**0.0001**
		[1] 147/55	23.89	+11.11	0.003	+7.85	0.0005
		[2] 141/15	23.74	+27.56	<.0001	+5.69	0.01

Trials were performed at 20˚C, with 10 µM FUdR. Control RNAi is the L4440 plasmid vector (negative control). Genotypes: N2 wild type, DR1786 *dpy-13(e184) unc-24(e138); mDp4 [unc-17(e245)] (IV;?). p,* log rank test. [C] combined data. [n] Trial number.